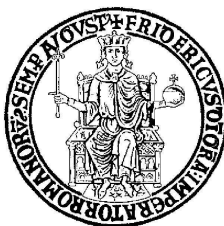


UNIVERSITÀ DEGLI STUDI DI NAPOLI “FEDERICO II”



DOTTORATO DI RICERCA IN BIOLOGIA APPLICATA
XXVI CICLO

Overexpression of Mash1 and Nurr1 synergize to induce the Dopaminergic Neuron Phenotype during *in vitro* differentiation of Neural Stem Cells

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1. INTRODUCTION

1.1 Midbrain dopaminergic neurons

Behind fundamental functions of the mammalian brain, such as fine motor integration, neuroendocrine hormone release, cognition, emotion, reward and memory resides the regulatory role of the catecholamine dopamine (DA). This neurotransmitter is produced and released by an anatomically and functionally heterogeneous class of neurons: the dopaminergic neurons.

In the mammalian central nervous system (CNS) dopaminergic nuclei have a broad distribution and are located in the area A16 of the olfactory bulbs (Gudelsky and Moore 1976) area A17 of the retina (Djamgoz and Wagner 1992), areas A11-A15 of the diencephalon [e.g. hypothalamic arcuate nucleus (A12; Kizer et al. 1976) , sub-parafascicular thalamic nucleus (A13;Takada 1993)] and areas A8-A10 of the ventral midbrain, where the most prominent source of DA in the CNS (Fig.1) is found.

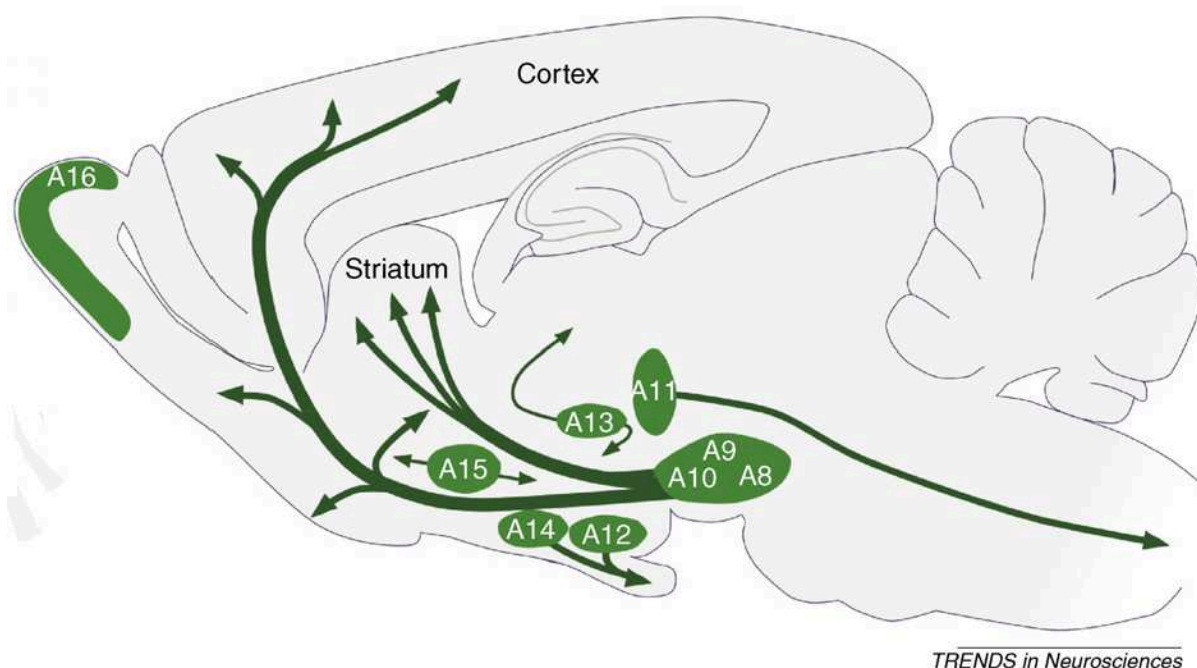


Figure 1. Schematic representation of dopaminergic nuclei in adult rodent brain. The scheme represents the disposition of main dopaminergic nuclei and their projections. A8, A9 and A10 nuclei are usually indicated as midbrain dopaminergic neurons with the nomenclature introduced by Dahlstrom and Fuxe in 1964 (Dahlström and Fuxe 1964). A8, Retrorubral field; A9, Substantia Nigra; A10, Ventral Tegmental Area. Image from (Björklund and Dunnett 2007)

The main dopaminergic nuclei in the midbrain (Mb) comprise the Retrorubral Field (RRF, area A8), the Substantia Nigra (SN, area A9) and the Ventral Tegmental Area (VTA, area A10) (Fig.2). The latter two give rise to two main circuits: the nigrostriatal pathway (NSp) and the mesocorticolimbic pathway (MCLp). The former is involved in the control of voluntary movement while the latter regulates superior cognitive ability such as reward, attention and emotional as well as certain aspects of mood control. The SN can be divided in the *pars compacta*, that contains DA cell bodies and the *pars reticulata*, located caudally, where are confined DA dendritic extensions connecting with GABAergic neuron *somata*.

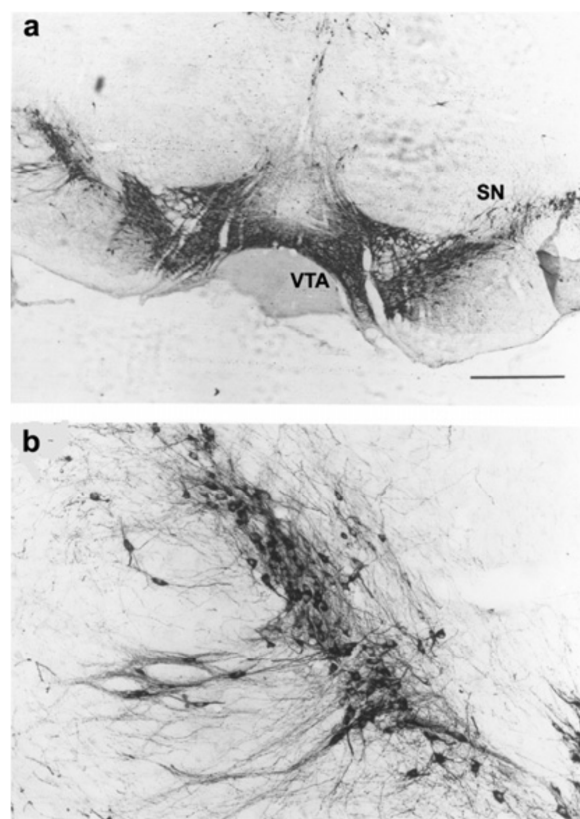


Figure 2. Tyrosine hydroxylase-immunostaining of mDA neurons in the adult mouse brain. (a) Anti-TH immunostaining of the ventral tegmental area (VTA) and the substantia nigra (SN) in the adult mouse ventral midbrain (b). A magnification of the substantia nigra. Image from (di Porzio et al. 1990).

In rodents, DA axons departing from *pars compacta*, reach the *striatum* (corresponding to *caudate-putamen* in humans), one of the principal basal nuclei, to form the ascending NSp. Basal nuclei are involved in motor control but do not connect directly with the spinal cord (Kandel et al. 2000).

Briefly, the striatum receives projections directly from the cortex and innervates the *thalamus* via the *globus pallidus* to return back to the cortex. This circuit includes a direct pathway and an indirect pathway, which includes the *subthalamic nucleus* and its function is to amplify activity schemes in the frontal cortex (Fig.3). At functional level the NSp represents the first relay of extrapyramidal tract, which controls non-volitional muscular tone, balance, initiation and velocity of movement (Lynd-Balta and Haber 1994).

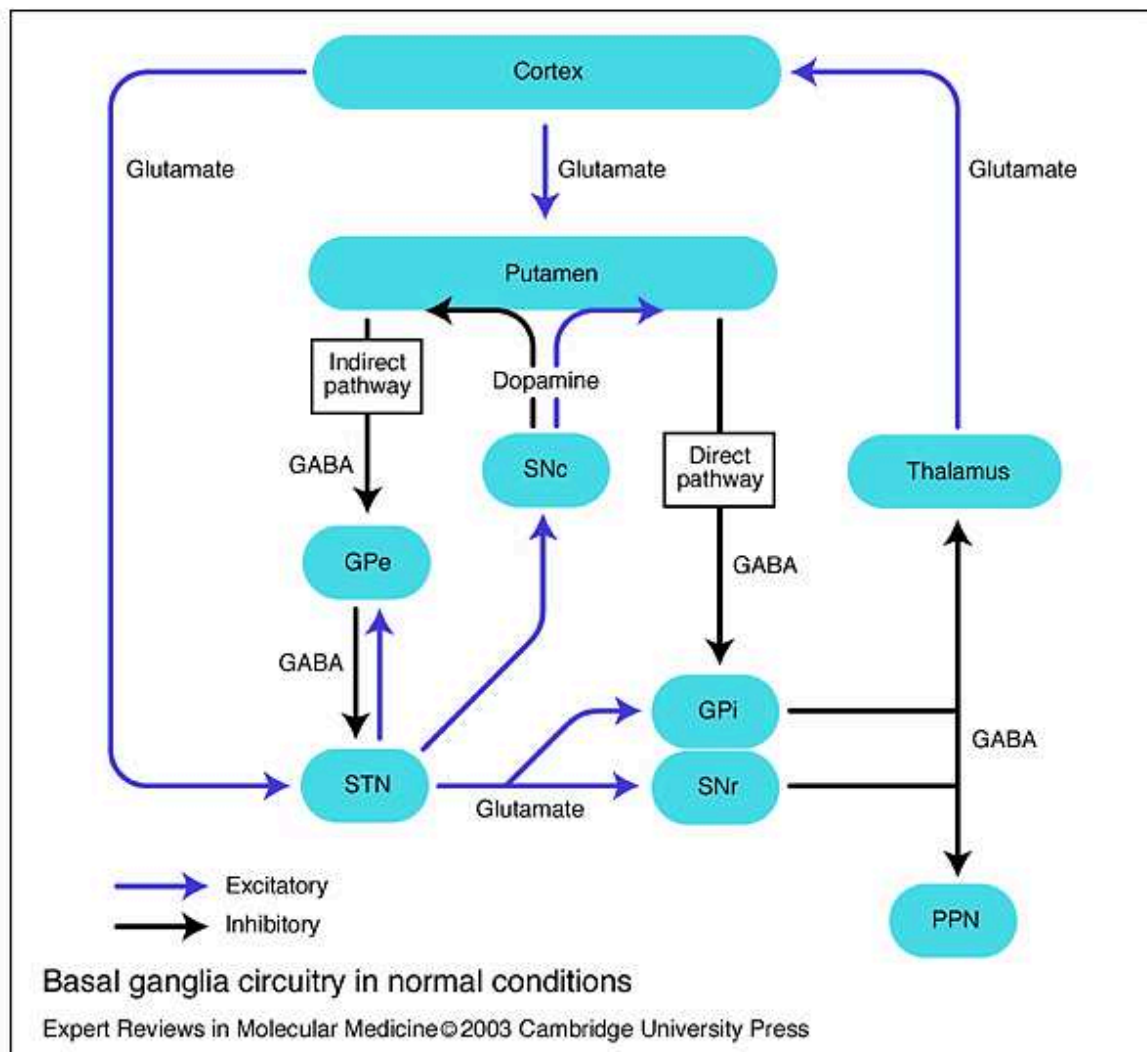


Figure 3. Extrapyramidal system network. It is shown the modulatory action of the *substantia nigra* on the caudate/putamen (corresponding to the striatum in rodents). In blue are shown glutamatergic excitatory pathways, black arrows indicate GABA inhibitory pathways. SNr, Substantia nigra *pars reticulata*; SNc, substantia nigra *pars compacta*; GPi, internal globus pallidus; GPe, external globus pallidus; STN, subthalamic nucleus; PPN, pedunculopontine nucleus.

Degeneration of nigrostriatal pathway leads to an over-function of inhibitory GABAergic circuit from the basal ganglia with subsequent decrease of the thalamic excitation of the prefrontal and primary motor cortex. The consequences are progressive loss of muscle control, tremor, rigidity and difficulty to complete simple motor tasks, a condition known in humans as the Parkinson's disease (PD; Thomas and Beal 2007), the syndrome described for the first time in 1817 by James Parkinson in his "Essay on the shaking palsy".

The VTA, organized in four primary nuclei, mainly projects to the limbic system (*nucleus accumbens*, *olfactory tubercle*), cortical areas (*prefrontal*, *cingulate* and *perirhinal cortex*) as well as *septum*, *amygdala* and *hippocampus* to form the mesocorticolimbic pathway (MCLp). The MCLp regulates different aspects of emotional behaviors, in particular mood control and reward related to natural pleasures like food, sex or social interactions (Wise 2004). Dysregulation of MCLp is linked to mood disorders (Zacharko and Anisman 1991; Martin-Soelch 2009), schizophrenia (Laviolette 2007), attention deficit hyperactivity disorder (ADHD; Ohno 2003) and drug addiction (Morales and Pickel 2012).

These two main dopaminergic pathways in the midbrain are not completely separated since their projections partially overlap. Indeed previous studies has been shown that the dysfunction of one can bring consequences on the function of the other (Jellinger 1991; Péron et al. 2012).

1.2 Molecular aspects of the dopamine biosynthesis

At biochemical level, DA is synthesized in the *soma* and in neuronal terminals from tyrosine. This amino acid is converted in *levodopa* (L-DOPA) by *tyrosine hydroxylase* (TH), the rate-limiting enzyme in catecholamine biosynthesis. The activity of this enzyme is regulated by *protein kinase cyclic adenosine monophosphate* (cAMP)-*dependent* (PKA). L-DOPA is then readily converted in DA by the ubiquitous enzyme *Aromatic L-amino acid decarboxylase* (AADC; Fig.4).

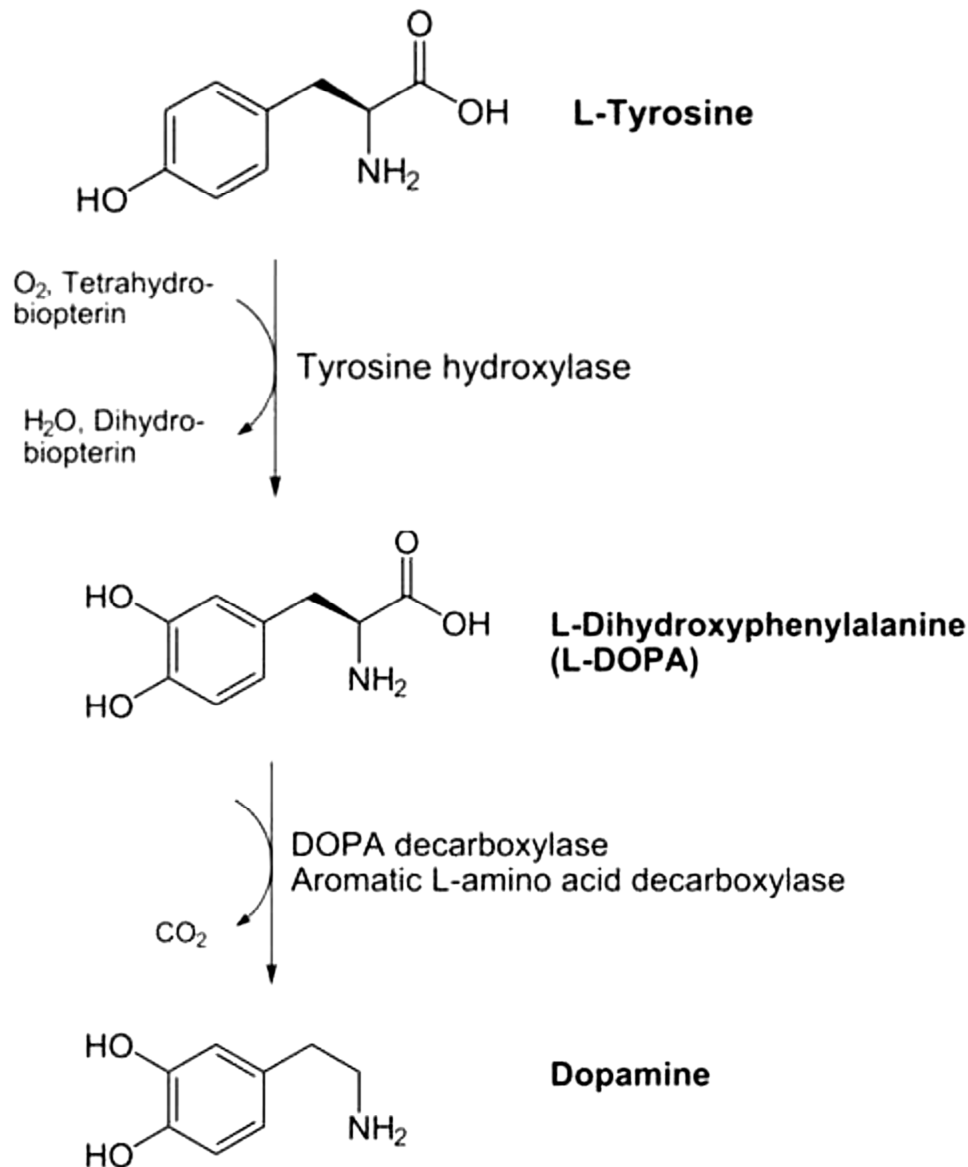


Figure 4. Dopamine biosynthesis. L-Tyrosine is converted in L-DOPA by tyrosine hydroxylase (TH) and in dopamine by aromatic L-amino acid decarboxylase (AADC).

DA is synthesized in the synaptic terminals and promptly stored in the specific nerve-endings organelles, called *dense core vesicles*, by the *vesicular monoamine transporter* (Vmat2), a 12 transmembrane domain protein. Upon the arrival of nerve impulse to the synaptic terminal, vesicles fuse with presynaptic membrane to release the DA in the synaptic cleft where it can bind 5 different postsynaptic receptors (DAR 1-5) to trigger a cascade of intracellular pathways that lead to the neuronal response. These receptors belong to the G-coupled receptor superfamily and are classified in two subfamilies: D1R (D1 and D5) and D2R (D2, D3 and D4).

The key difference between them concerns the cascade that they trigger: while D1Rs lead to an increase in cAMP, D2Rs are negatively coupled with the adenylate cyclase determining a decrease in cAMP. Both families act by modulating the cAMP/PKA transduction pathway to regulate the intracellular levels of Ca^{2+} . The released DA can act on receptors that are distant from its release site, a modality of communication called paracrine transmission. DA receptors have a peculiar anatomical and cellular distribution. The D2 receptors, for instance, are localized on the post-synaptic membrane, but also on the DA neurons membranes (autoreceptors) and act by feedback inhibition of DA transmission, regulating DA release (Ford 2014).

DA in the synaptic cleft is metabolized to *homovanillic acid* (HVA) by *catechol-O-methyl transferase* (COMT) or is degraded to *3,4-dihydroxyphenylacetic acid* (DOPAC) by the monoamine oxidase enzyme (MAO). Neurotransmission is terminated mainly by a process named *high affinity uptake* by which the released DA is actively captured back into the presynaptic endings through the membrane glycoprotein *dopamine transporter* (DAT). Once back in the cytoplasm, DA can be re-stored in vesicles or degraded by mitochondrial MAO in DOPAC. A schematic activity of dopaminergic transmission is showed in Fig.5.

DAT, also known as SLC6A3, is a 12 transmembrane domain $\text{Na}^+\text{-Cl}^-$ dependent transporter, whose importance is highlighted by the observation that DAT knock-out mice show spontaneous hyperlocomotion due to increased persistence of DA in the synaptic cleft. DAT is also target of several drugs such as cocaine and amphetamine, indeed previous data show the insensibility of DAT-null mice to their administration (Thomsen et al. 2009).

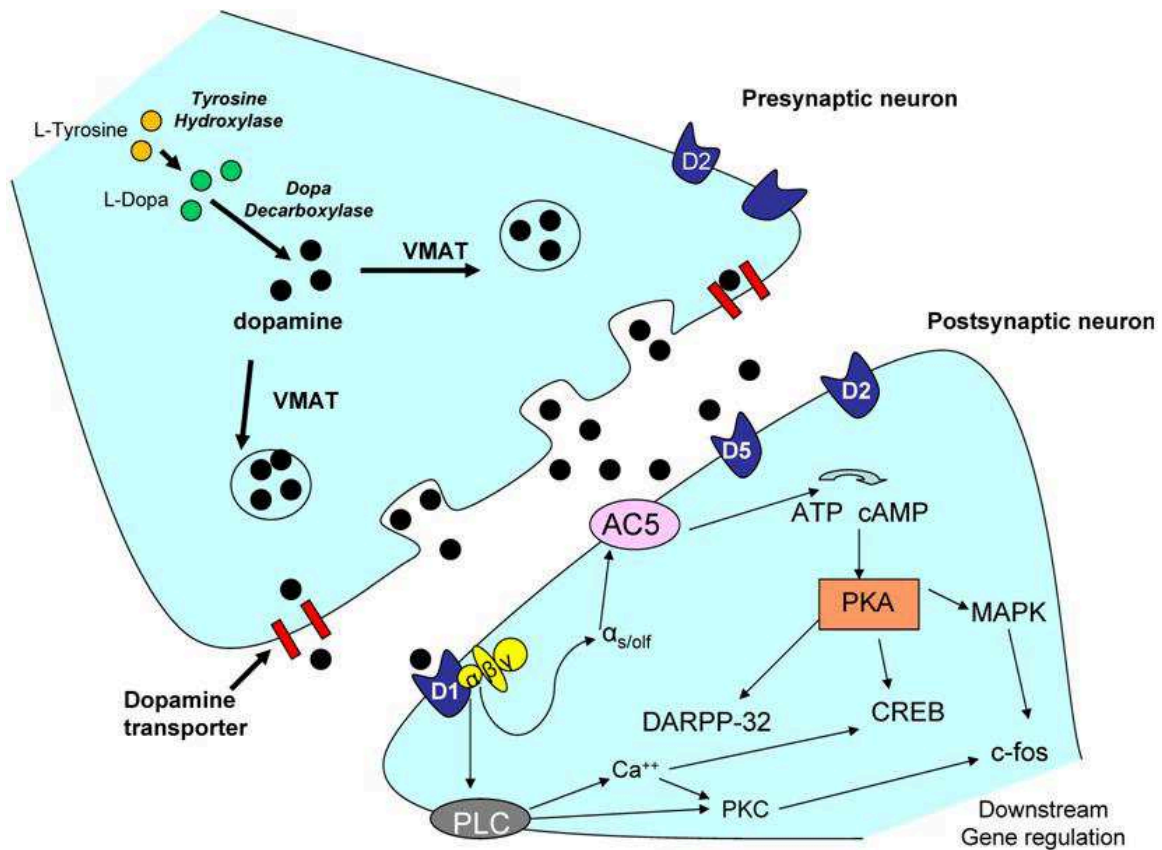


Figure 5. Schematic representation of dopaminergic synapse. Image shows the synthesis, the storage and the release of dopamine with the activation of receptors and downstream intracellular cascade. DA in the synaptic cleft is taken up by DAT to be restored in vesicles or degraded by COMT and MAO (not shown). Image from (Knab and Lightfoot 2010).

1.3 Molecular and transcriptional control of dopaminergic neurons development

The achievement of phenotypic specificity of midbrain DA (mDA) neurons is a multi-step process orchestrated by cell-intrinsic factors as well as environmental cues that define an accurate space-time sequence. In this way, a multipotent progenitor is subjected to a sequential transcriptional activation that follows an accurate genetic program.

The time course of development of mDA neurons can be broadly divided into 4 stages: early midbrain patterning; induction and specification of dopaminergic precursors; differentiation of post-mitotic DA neurons; functional maturation of mDA neurons (Fig.6; Abeliovich and Hammond 2007; Perrone-Capano et al. 2008).

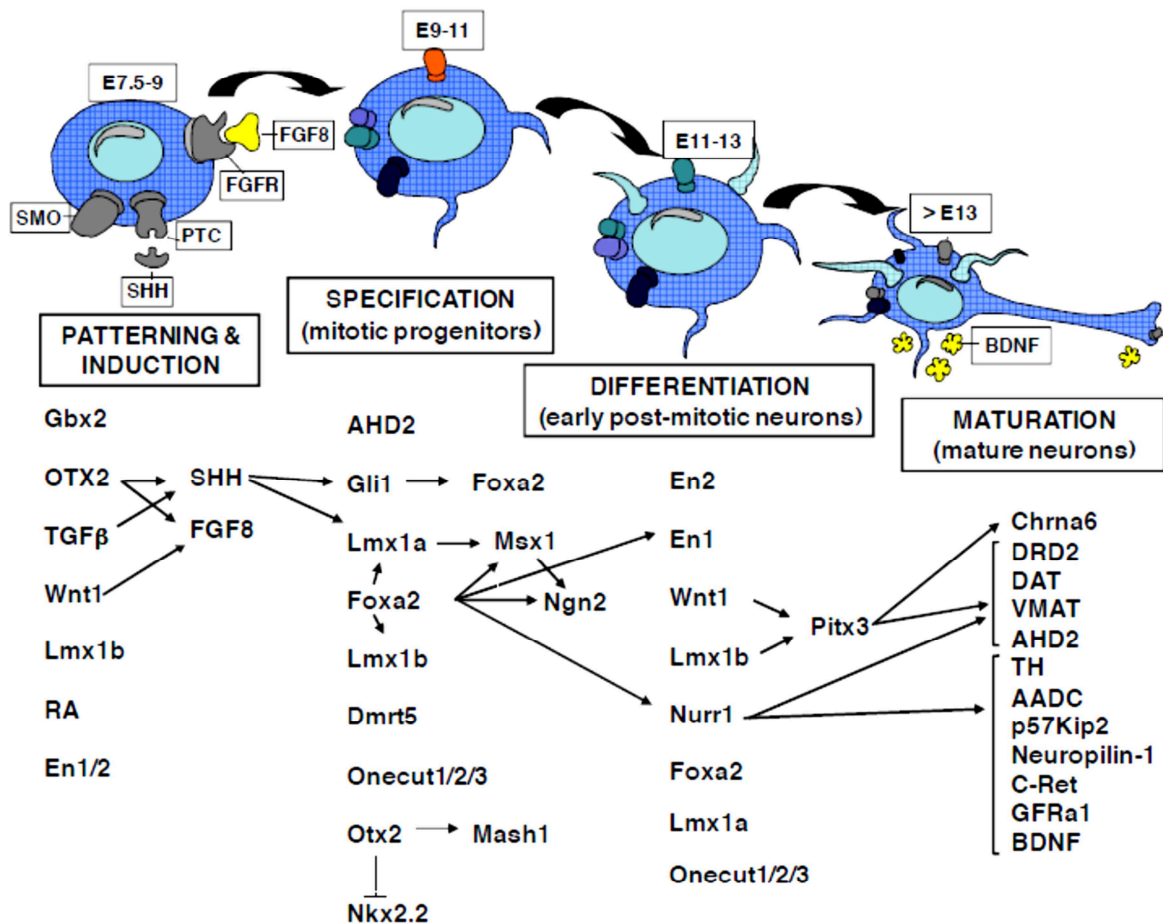


Figure 6. Midbrain dopaminergic neurons differentiation. Diagram shows the sequential activation of transcription factors during the time course of embryonic development. Arrows indicate possible interactions between the molecules.

1.3.1 Early midbrain patterning

Regionalization of the midbrain begins early in neural plate development (E7.5) with the definition of molecular borders that define the positioning of key signaling centers: the floor plate, present at the ventral midline, and the midbrain-hindbrain boundary (MHB, also known as isthmus organizer) at the caudal extreme of developing mesencephalon. Loss of function studies have identified a number of transcription factors (TFs) involved in the early patterning of midbrain. For example Otx2 and Gbx2, whose expression is mutually exclusive, are required for the correct positioning of MHB. Otx2, a member of the Otx1 family, plays a remarkable role in specification and regionalization of anterior structures such as forebrain and midbrain (Simeone et al. 2002). On the other hand, Gbx2 is expressed more posteriorly and it is essential for the correct development of midbrain and cerebellum.

Chick/quail transplantation experiments have highlighted the interplay between these two factors showing that the posterior limit of Otx2 marks the anterior limit of Gbx2 expression, circumscribing the isthmus (Hidalgo-Sánchez et al. 1999). Moreover, cell transplantation studies shows that also Wnt8 is required for the initial subdivision of the neuroectoderm, including onset of posterior Gbx1 expression and establishment of the posterior border of Otx2 expression (Rhinn et al. 2005). Among the other molecules, retinoic acid (RA) is required for the proper positioning of the midbrain–hindbrain border and is therefore indirectly needed for the correct organization of the midbrain (Holder and Hill 1991; Avantaggiato et al. 1996); Wnt signaling (Wnt1 and Wnt5A) is crucial for the establishment of the midbrain–hindbrain region and is involved in activating engrailed (En) genes, which are also necessary for later stages of mDA neuronal development (Castelo-Branco et al. 2003; Smidt and Burbach 2007).

A key feature of any signaling center is that it must provide a diffusible signal that can be received by a group of cells, which in turn may be “primed” through transcription of cell-specific genes. As described below, the MHB exerts its inductive role by secreting the *fibroblast growth factor 8* (FGF8), a morphogen that drives the correct regionalization along the anterior-posterior axis of the developing CNS. The FP, conversely, secretes the lipid-modified glycoprotein Sonic hedgehog (SHH), which is known for its pivotal role in the specification of the different ventral populations along the dorso-ventral axis.

1.3.2 Induction and specification of dopaminergic precursors

Early patterning events specify a permissive region in which DA progenitors cells are exposed to inductive signals in a position-specific manner. In this way, gradients of extracellular inductors, together with cell-intrinsic responses, establish a Cartesian grid able to direct cells toward the specific fate depending on the cell position in the grid. Thus, two orders of molecular events can be recognized in the induction of DA phenotype: first, signaling centers secrete diffusible factors along the dorso-ventral

and rostro-caudal axes of the neural tube that impart positional information; concurrently, cell-intrinsic factors within mesencephalic progenitors progressively restrict cell fate choice. As pointed out above, SHH, produced by the FP, and FGF8, secreted by the MHB, are able to cooperate for mDA induction before E9.5 in mice (Hynes and Rosenthal 1999)(Fig.7).

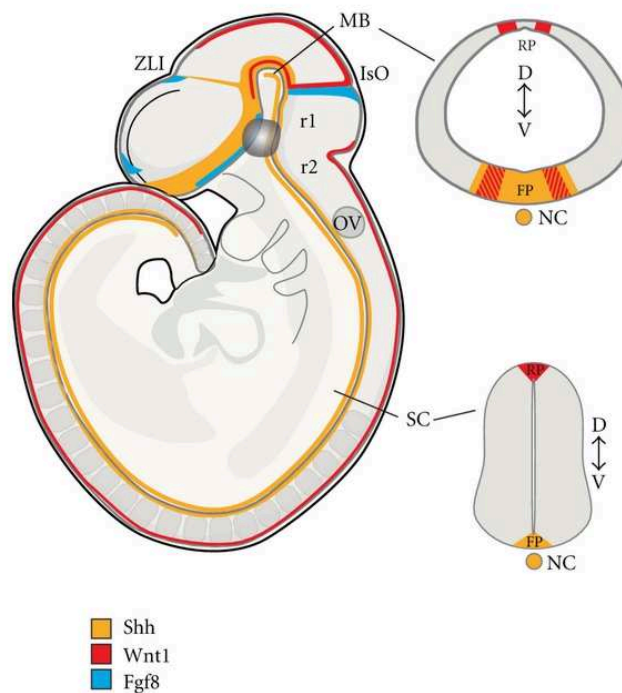


Figure 7. Specification of DA neurons by SHH and FGF8 gradients. Sagittal and coronal views of the midbrain and spinal cord of the mouse embryo showing the expression patterns of Shh, Wnt1 and Fgf8, that pattern the neural tube along rostro-caudal, dorso-ventral at E9.5. FP: floor plate; IsO: isthmus organizer; MB: midbrain; NC: notochord; OV: otic vesicle; RP: roof plate; SC: spinal cord; ZLI: zona limitans intermedia. Image from (Aguila et al. 2012).

Several studies show how ectopic expression of SHH and FGF8 is able to induce mDA neurons. Indeed DA neurons failed to develop after silencing of endogenous SHH with a neutralizing antibody as well as after FGF8 neutralization indicating that both factors are needed (Roussa and Krieglstein 2004). *In vitro* experiments also underline the importance of both factors as inducers of DA fate in neural stem cells line (Kim et al. 2003c) and in primary cultures (Volpicelli et al. 2004a).

SHH, the most studied member of the *Hedgehog* family is initially expressed in the notochord and then in the floor plate.

The SHH protein is initially synthesized as a 45-kDa precursor protein that undergoes auto-proteolytic cleavage leading to an amino-terminal cleavage product of approximately 20 kDa (referred to as SHH-N) which is responsible for the induction of the floor plate at the ventral midline of the neural tube. In addition, this peptide is secreted by the notochord and floor plate and, as already pointed, encodes a ventralizing activity, thereby inducing ventral cell types (Hynes et al. 1995; Roussa and Kriegstein 2004). SHH acts as secreted morphogen through the binding with his specific receptor *Patched1* (Ptc1) (Stone et al. 1996). The latter acts *via* the inhibition of the 7-transmembrane protein *Smoothened* (Smo) which is released after the binding of SHH. Smo triggers an intracellular cascade responsible for the activation of the zinc-finger proteins Gli (Gli1, Gli2 and Gli3), the key downstream mediators of the SHH response in the vertebrate neural tube (Hynes et al. 1997).

During early steps of induction, several proteins can be detected to characterize mDA progenitors. One of the first molecules induced is the *aldehyde dehydrogenase 2* (Ahd2; expressed at E8.5) which is one of the major players in *retinoic acid* (RA) synthesis (Wallén et al. 1999). This observation allows to assume that RA may have a role not only in MHB positioning but also in neuronal fate specification.

The above cited Otx2 also seems to have a role in early stages as Otx2-null mice fail to generate DA neurons (Omodei et al. 2008). Indeed, Otx2 exerts its action also by inhibiting the negative regulator of mDA fate Nkx2.2 (Prakash et al. 2006). Another function of Otx2 is to induce *proneural* genes such as Ngn2 and Mash1 (Ascl1) which are required in mitotic DA progenitors as well as in later stages (Vernay et al. 2005).

The LIM-homeobox gene Lmx1a, which is expressed around E9 in mice and is induced by SHH, plays an important role. Its overexpression is capable to ectopically induce mDA neurons both *in vivo* and *in vitro* (Millonig et al. 2000; Sánchez-Danés et al. 2012) as well as its silencing leads to a severe loss of the latter (Andersson et al. 2006b). Notably, Lmx1a controls the expression of the homeodomain factor Msx1, a transcriptional repressor that inhibits expression of negative regulators of neurogenesis, such as the homeodomain factor Nkx6.1 (Nakatani et al. 2010).

Finally, this cascade induces broadly proneural factors such as Ngn2, which is necessary for the normal mDA generation and maturation.

Together with Lmx1a, Lmx1b is also required in the initial steps of ventral mDA neurons specification (Andersson et al. 2006b). They are indeed co-expressed in developing midbrain and have a cooperative role in early and late phases of mDA neurons development by regulating proliferation, specification, and differentiation (Yan et al. 2011). Remarkably, Lmx1b can also induce ectopic DA neurons (Nakatani et al. 2010) and its action can partially overlap with Lmx1a, as showed by elegant experiments on the Lmx1a mutant. Indeed in this model only 46% of mDA neurons is lost suggesting that Lmx1b can partially compensate the loss of Lmx1a (Ono et al. 2007). In a recent work, Deng and coworkers suggest that Lmx1b is required during the specification phase and influences the differentiation of several neuronal subtype in the midbrain, included ocular *motor neurons* and *red nucleus neurons*, while Lmx1a functions seem to be more restricted to the mDA fate (Deng et al. 2011).

Strictly related with Lmx1a and b expression is the role of Foxa1 and Foxa2 transcription factors. Indeed, a study of 2010 revealed that Lmx1a can specify mDA fate only within Foxa2⁺ mesencephalic progenitors and, conversely, the supportive action of Lmx1a/b through the repression of Nkx6.1 is required by Foxa2 to specify FP identity (Nakatani et al. 2010). Foxa1 and Foxa2 (Foxa1/2) are members of the Foxa family of forkhead/winged helix transcription factors, which have diverse roles in regulating development, tissue homeostasis and tumorigenesis (Hannenhalli and Kaestner 2009). They have been strongly associated with multiple phases of mDA neurons development, influencing the cell fate and the expression of other dopaminergic-related molecules in a dosage-dependent manner (Ferri et al. 2007). Analysis of the double mutant for both genes has revealed a positive effect on the expression of neurogenic factors such as Ngn2 and Mash1 in the specification stage while, later in development, they are able to regulate Nurr1 and En1 in early post-mitotic neurons and AADC and TH. In particular the binding of Foxa2 with the TH promoter was proven by ChIP experiments (Ferri et al. 2007; Lin et al. 2009). The role of Foxa2 in the specification and the maturation of mDA neurons was

demonstrated also *in vitro* by observing the increase in the DA population after its co-overexpression with Nurr1 (Lee et al. 2010).

Moreover, a genome-wide analysis of its targets revealed that Foxa2 is able to bind enhancers of floor plate genes, like Shh and Ferdl3 and promotes midbrain identity upregulating the expression of DA genes such as Lmx1a, Lmx1b, Msx1 and repressing factors involved in the specification of ventro-lateral fates like Helt, Tle4, Otx1 and Sox1 or also components of the SHH signaling (Metzakopian et al. 2012). Interestingly, mice carrying one copy of the Foxa2 gene show abnormalities in motor behavior in old age associated with a progressive loss of dopamine neurons, a feature of PD (Kittappa et al. 2007).

Recent studies identified new players in the specification of mDA progenitors such as the *doublesex and mab-3-related transcription factor5* (Dmrt5), which has been shown to regulate the expression of Foxa2, Lmx1a and Msx1 (Gennet et al. 2011), and the factors belonging to the Onecut family. In particular Single and double mutants for Onecut1 and Onecut2 showed that the extension and the distribution of TH⁺ neurons is affected in their absence, but to date their function still needs to be determined (Chakrabarty et al. 2012).

1.3.2.1 Role of Neurogenin2 and Mash1

The transcription factors belonging to the basic Helix-Loop-Helix (bHLH) gene family are known to regulate crucial developmental processes in various mammalian tissues. Neural bHLH transcription factors such as Neurogenin 1 (Ngn1), Ngn2, NeuroD, and Mash1 are expressed in both the central and the peripheral nervous systems during development and promote early neuronal differentiation (Kageyama et al. 1997; Bertrand et al. 2002). In the context of the midbrain development, Ngn2 and Mash1 (also known as Ascl1) play a remarkable role. Both factors act forming a heterodimer with an ubiquitously expressed bHLH factor, E47, and activate gene expression by binding to a characteristic sequence called E-box (CANNTG)(Kageyama et al. 2005).

Ngn2 is a *proneural* gene related to the *Drosophila* factor *atonal* that is positively regulated by Msx1 during midbrain development (Fode et al. 1998; Andersson et al. 2006b). Within the ventral midbrain (VM), Ngn2 expression is restricted to the ventricular zone (VZ), and its expression correlates both spatially and temporally with the generation of mDA neurons (Thompson et al. 2006). This gene has indeed been showed to be essential for the proper development of mDA neurons since its absence impairs and delays their differentiation. However the initial loss of DA neurons is partially recovered in later stages of development, probably due to a compensatory action of Mash1. Moreover, loss of function studies revealed that Ngn2 is required for the differentiation of ventricular zone progenitors into post-mitotic DA precursors (Andersson et al. 2006a; Kele et al. 2006). Transplantation studies show how Ngn2+ cells are able to engraft into host animals where they give rise to TH+ neurons (Thompson et al. 2006). Interestingly the ectopic expression of Ngn2 and Nurr1 in neural progenitors regulates the differentiation of mDA neurons (Park et al. 2008).

Different studies show that also the murine homolog of *achete-scute* Mash1 is expressed in a complementary pattern with Ngn2 in the developing murine VM where both play an important role in regulating neurogenesis (Kele et al. 2006; Kim et al. 2007; Nelander et al. 2009). By generating mutations in mice in which the coding sequences of Mash1 and Ngn2 were swapped, researchers demonstrated that Mash1 and Ngn2 have divergent functions in specification of neuronal subtype identity, with Mash1 harboring the characteristics of an instructive determinant whereas Ngn2 functions behaving as a permissive factor that must act in combination with other factors to specify neuronal phenotypes (Parras et al. 2002). Mash1 plays a role at early stages of development of specific neural lineages in most regions of the central nervous system and of several lineages in the peripheral nervous system, in particular telencephalon, including ventricular zone (VZ) of the developing olfactory bulb (OB) and ganglionic eminences, diencephalon, midbrain, spinal cord, and retina (Guillemot and Joyner 1993). Indeed, loss of function and chromatin immunoprecipitation (ChIP) experiments showed that Mash1 regulates the expression

of *Delta-like1* (*Dll1*), a strong inducer of neuronal fate (Casarosa et al. 1999; Castro et al. 2006; Yun et al. 2010). One of the principal features of Mash1 is to coordinate the program of neurogenesis by controlling the progression of neural progenitors through the successive phases of proliferation, cell cycle exit, and differentiation (Castro et al. 2011).

Evidence that Mash1 has a role in the specification of neuronal identity has been obtained in different regions of the nervous system. For example, Mash1 can induce expression of GABAergic neuron markers such as *Dlx1* and *GAD67*, in the cerebral cortex (Parras et al. 2002). It has also been reported that a cooperative or synergistic action with homeodomain factors specifies the subtype identity of particular neurons, for example Mash1 with *Phox2b* specifies the ventral hindbrain motor neurons (Dubreuil et al. 2002; Stanke et al. 2004).

The expression of Mash1 is induced in the midbrain by *Otx2* around E10 (Vernay et al. 2005), however its expression is also controlled by RA and by the Notch pathway (Jacob et al. 2013). Recent studies underline a role for Mash1 in the specification of DA phenotype, for example forced expression of Mash1 and *Nurr1* in cortical progenitors caused a dramatic increase in morphological differentiation of DA cells (Park et al. 2006a; Park et al. 2006b; Kim et al. 2007). Elegant studies on the role of bHLH in the differentiation of DA lineage, have shown that *Ngn2* suppresses *Nurr1*-induced gene transcription in mDA neurons and that Mash1, which is expressed in *Nurr1*-positive young mDA neurons, stimulates mDA neuronal maturation independently from the activation of terminal differentiation markers such as *Pitx3* (Park et al. 2006a). The co-expression of Mash1 and *Nurr1* produce similar results also in adult neural stem cells, leading to the functional differentiation of DA neurons, able to survive, once transplanted, into hemi-parkinsonian rats (Shim et al. 2007). Moreover ectopic expression of Mash1 with specific factors (*Brn2*, *Myt1l*) is sufficient to reprogram fibroblasts into neurons (iN; Vierbuchen et al. 2010) while its co-expression with *Lmx1a* and *Nurr1* reprograms fibroblasts into functional DA neurons (Caiazzo et al. 2011). These experiments suggest a role of Mash1 in the “priming” of these cells to reprogram their fate.

A very recent paper seems to clarify the general mechanism of action of Mash1: it acts as a “pioneer” factor recognizing a particular chromatin configuration, called *trivalent state*, in its target promoters; once the bHLH factor binds these regions, it allows the acetylation of histones and the access of other transcription factors to the promoter (Wapinski et al. 2013).

1.3.3 Differentiation of post-mitotic mDA neurons

As shown by ³H-thymidine incorporation studies, the transition of mDA progenitors from the proliferative state to post-mitotic immature neurons happens, in mice, around E9.5-E13.5 as they exit the cell-cycle and migrate radially from the ventricular surface (Bayer et al. 1995; Abeliovich and Hammond 2007). In this stage, radially migrating cells start to express TH, an event that occurs early (E9.5 in mouse) as suggested by the observation of the first TH⁺ cells and fibers close to the ventricular ependymal layer (di Porzio et al. 1990).

Several key transcription factors, such as Engrailed (En) 1 and 2, Nurr1, Pitx3 and Lmx1b are involved in the development of post-mitotic mDA neurons. Here the expression of these factors is restricted to the early mDA neurons, suggesting that the factors function cell-autonomously. However, none of these transcription factors appear sufficient individually to instruct the mDA phenotype, suggesting a network model (Abeliovich and Hammond 2007).

1.3.3.1 Nurr1 and Pitx3

The *Nuclear Receptor Related 1 protein* Nurr1, also known as Nr4a2, is a member of the steroid-thyroid hormone receptor superfamily. It is expressed predominantly in the CNS in limbic areas and the ventral midbrain, including dopamine neurons (Zetterström et al. 1996). Nurr1 is currently considered an *orphan nuclear receptor*, since no ligands have been yet identified (Maruyama et al. 1998).

The current hypothesis is indeed that Nurr1 acts in absence of a ligand and can promote signaling via its heterodimerization partner, the retinoid X receptor (RXR; Perlmann and Wallén-Mackenzie 2004).

The onset of Nurr1 expression in the ventral midbrain occurs at E10.5, one day before the appearance of the dopaminergic marker enzyme, TH, at E11.5 (Volpicelli et al. 2004b) and persists in the adult brain.

Gene inactivation studies showed that Nurr1 is essential for the later stages of mDA cell development, as its loss results in a specific agenesis of midbrain DA neurons. However, later in development, Nurr1 is expressed in a wide range of brain areas, where its inactivation does not seem to affect other cell types (Zetterström et al. 1996; Saucedo-Cardenas et al. 1998). According with these studies, Nurr1 is a direct regulator of a number of key DA specific genes such as TH (Kim et al. 2003b), DAT (Sacchetti et al. 2001), Vmat2 (Colebrooke et al. 2006) and c-Ret. The latter encodes for the co-receptor of the *glial cell line-derived neurotrophic factor* (GDNF) family associated to the GDNF family receptor alpha (GFR α ; Wallén et al. 2001). Moreover, other data suggest that Nurr1 is involved in the responsiveness of mDA neurons to trophic factors by positively regulating *brain-derived neurotrophic factor* (BDNF; Volpicelli et al. 2007). Supporting the central role of this factor in the maturation of DA neurons, its overexpression is able to induce TH expression in NSC (Kim et al. 2003c; Shim et al. 2007; Soldati et al. 2012), in ESC (Martinat et al. 2006; Abasi et al. 2012) and in immortalized cell lines (Castro et al. 2001).

Defects in Nurr1 expression are associated with pathological states that affect mDA neurons: chronic cocaine administration downregulates Nurr1 in human and rat (Bannon et al. 2004; Leo et al. 2007). Notably, rare forms of familial PD are associated with mutation of Nurr1 which, in turn, has been found decreased in PD patients (Le et al. 2003; Sleiman et al. 2009; Decressac et al. 2013). Based on these evidence, recent studies are attempting to exploit Nurr1 potential in transplantation cell therapy for PD (Wagner et al. 1999; Kim 2011; Tan et al. 2012).

The bicoid-related homeodomain *Pituitary homeobox 3* (Pitx3) is another important determinant of mDA neurons identity. Its expression (E11.5 in mouse) is detectable in different brain areas during development but after birth is present only in mDA neurons where is maintained also during adulthood (Smidt et al. 1997). Data derived from the analysis of aphakia mutant mice (AK; which are Pitx3^{-/-}) suggested that Pitx3 is specifically involved in the terminal differentiation and/or early maintenance of mDA neurons, as these neurons are absent in AK animals (Smidt et al. 2004; Smidt and Burbach 2007). Additional studies on Pitx3-null mice assessed that it is required during the transition of post-mitotic mesencephalic DA progenitors to TH⁺ neurons. Nevertheless there is a difference in the timing of Pitx3 expression between SN, where its expression precedes TH and VTA where they are expressed simultaneously (Maxwell et al. 2005). These data identify ontogenetically distinct subpopulations of dopaminergic cells within the ventral midbrain based on the temporal and topographical expression of Pitx3 and TH. A recent work identified a developmental cascade in mice in which Ahd2, a major player in RA synthesis, is under the transcriptional control of Pitx3. These data position Pitx3 centrally in a mDA developmental cascade, linking this transcription factor to RA signaling (Jacobs et al. 2007). Other studies underline the interplay between Pitx3 and Nurr1 since chromatin immunoprecipitation experiments identified Vmat2 and DAT, known Nurr1-regulated genes, as Pitx3 targets (Hwang et al. 2009). On the other hand Pitx3 modulates the Nurr1 transcriptional complex by decreasing the interaction with SMRT, which acts through HDACs to keep its target promoters in a repressed deacetylated state (Jacobs et al. 2009).

A recent study, performed in our laboratory showed a direct regulation of Pitx3 expression by Nurr1. In particular, Nurr1 can regulate Pitx3 expression through its binding to a non-canonical NBRE element located 220bp upstream the transcription initiation site on the Pitx3 promoter (Volpicelli et al. 2012).

1.3.3.2 En1 and En2

Engrailed-1 (En-1) and En-2 are initially expressed during early development in a domain encompassing the posterior midbrain and anterior hindbrain where are required for the generation of MHJ (Hidalgo-Sánchez et al. 1999; Liu and Joyner 2001). Their expression is no longer detectable until E11.5, when En1 and En2 start to be expressed again in ventral mDA differentiating neurons (Simon et al. 2001; Albéri et al. 2004). They are cell-autonomously required to prevent apoptosis of these neurons towards the end of embryonic development, suggesting a key role for both genes in the regulation of mDA neuron survival and death, a function that may endure also during postnatal development and maintenance (Albéri et al. 2004). Interestingly, the En1^{+/-} heterozygote mice show an adult phenotype that resembles key pathological features of PD. Specifically, postnatal mutant mice exhibit a progressive degeneration of dopaminergic neurons in the SN during the first 3 month of their lives, leading to diminished storage and release of dopamine, motor deficits and a lower body weight (Sgadò et al. 2006).

1.3.4 Maturation and survival of mDA neurons

Functional maturation of newly born mDA neurons takes place as they extend axonal and dendritic processes that must be correctly targeted to their destinations and, at the same time, become capable to express molecules involved in DA synthesis, storage and high-affinity uptake. The activation of genes involved in DA neurotransmission takes place at different time in development under the control of various environmental cues, following the early commitment of DA neuroblasts (Perrone-Capano and di Porzio 1996; Perrone-Capano and di Porzio 2000; Perrone-Capano et al. 2008). The axonal outgrowth to the target and the functional maturation are not separate processes as has been previously showed that interactions of presynaptic mDA neurons with striatal target neurons play an important role, at least *in vitro*, in promoting key aspects of DA neurotransmission (Prochiantz et al. 1979; di Porzio et al. 1980).

Studies focused on the identification of guidance factors and cues have described the events that unfold during mDA axonal pathfinding, synapse formation and stabilization. EphrinB2, involved in axonal guidance, and its receptor EphB1 can regulate the correct targeting of nigrostriatal neurons, with EphB1 expressed mainly in SN, and EphrinB2 expressed in the striatum (Yue et al. 1999). Notably, addition of EphrinB2 in primary mesencephalic rat cultures can lead to an upregulation of Nurr1 transcript (Calò et al. 2005). Conversely, Nurr1 has been showed to increase expression of Neuropilin1, a co-receptor for the semaphorin family ligands (Hermanson et al. 2006). Slits and Netrins may also be involved in target recognition by nigrostriatal mDA neurons since in explant cultures it was clearly shown that guidance of mDA axons can be regulated through the Netrin1 and Slit2 pathway. Consistent with this notion, mDA neurons express the Slit receptors, Robo1 and Robo2, and the Netrin1 receptor DCC (Lin et al. 2005). Interestingly, DCC-null mutants show profound alterations in DA circuitry, including migration defects of DA progenitor cells and reduced numbers of DA cells in Mb nuclei (Xu et al. 2010). The importance of Slit/Robo pathway is underlined by double knock-out studies highlighting guidance errors during the mDA pathfinding in the ventral regions within the diencephalon (Dugan et al. 2011).

Upon target innervation, mDA axons likely compete to establish functional synapses and survive. In particular, a wave of apoptotic mDA cell death is observed perinatally in rodents with an initial peak on postnatal day (P)2, and a second on P14 (Burke 2003). Strictly connected with these events is the role of target-derived trophic factors, along with autocrine and paracrine signals involved in the maintenance and survival of these cells. The role of many trophic factors has been identified in mDA neuron maintenance such as the well-described GDNF (Lin et al. 1993; Beck et al. 1995), the TGF- β (Farkas et al. 2003), BDNF (Hyman et al. 1991) and the recently identified *conserved dopamine neurotrophic factor* (CDNF; Kriegstein 2004; Lindholm et al. 2007). Notably, GDNF and BDNF have been show to promote the survival and the differentiation of mDA neurons (Hyman et al. 1991; Consales et al. 2007) along with a positive effect on their arborization (Costantini and Isacson 2000).

As pointed above, BDNF is in part regulated by Nurr1, suggesting the existence of a Nurr1-BDNF loop protecting mDA during development and, possibly, in the adulthood (Volpicelli et al. 2007). According with this observation, BDNF-null mice show defects in the proper development of DA dendrites in the SN, although they have a normal asset of mDA neurons. This defect becomes evident only in the postnatal period and defines a role of BDNF in the phenotypic maturation (Baquet et al. 2005).

In conclusion, lack or limited production of DA-associated trophic factors may play an important role in the pathogenesis, as well as in the potential treatment, of neurological diseases associated with DA neurons.

1.4 Stem cell models to study mDA neurons

In the last decades, the use of a number of cytokines and growth factors, and the discovery of stem cells in the embryonic and adult CNS has been exploited to generate DA neurons, expand DA neuron precursor populations and increase DA phenotype *in vitro*. These efforts raised the opportunity to have a simplified model *in vitro* that recapitulate, at least in part, the development of mDA neurons to analyze intrinsic and extrinsic cues involved in the DA fate, with the final aim to understand and possibly cure pathological states arising from DA system disruption.

1.4.1 Stem cells

After the bombing of Hiroshima and Nagasaki in 1945, more than 140.000 people died immediately but many others developed severe leukemia and total loss of the bone marrow due to massive radiation exposition (Jacobson et al. 1951). Just a few years later hematologists discovered a population of cells in the bone marrow capable of replacing all the blood cells (Nowell et al. 1956; Ford et al. 1956). These cells were later identified as stem cells.

Stem cells are undifferentiated cells that can be toti-, pluri- or multipotent. During the embryonic development, they are the founder cells of every tissue, organ and cell in the body of animals and plants, while in the adulthood they constitute a sort of repair system of the body and can divide without limit to replenish other cells as long as the animal or plant is alive. Key features that define a stem cell are the ability to self-renew through cell division (asymmetric division) and the competence to generate specialized cell types through differentiation. The asymmetric division is a distinctive biological characteristic, which distinguishes the stem cells (or multipotent progenitors) from all other cell types where the mitotic division is symmetrical (Bellenchi et al. 2012). As they differentiate to give rise to different types of cells in various tissues, their potential becomes more restricted and, at the same time, their number decreases. Nevertheless, in the adult various organs retain a stem cell compartment, located in a specific microenvironment, called *niche*, which regulates their behavior (Doetsch 2003a). Generally, adult stem cells are uni- or multipotent as they give rise only to cell lineages required by the specific tissues containing their niche. Here, stem cells may be present in different proliferative state depending on the organ in which they are present. For example, niches show active proliferation in tissues that require a high cell turnover, such as the blood, the epidermis, the intestinal epithelium and male gonads while in tissues where cell loss is limited, such as the liver, the teeth and the brain, the stem cells are present in a quiescent or low proliferation state. However, in these tissues proliferation can be activated by physiological and pathological stimuli (Bellenchi et al. 2012).

Very recent studies opened the interesting perspective of generating stem cells from adult somatic cells. The so called *induced pluripotent stem cells* (iPSC) are obtained by overexpressing into skin fibroblasts a set of transcription factors able to “reprogram” the cell to a pluripotent state (Takahashi and Yamanaka 2006; Yamanaka and Blau 2010). On the general wave of enthusiasm generated by this discovery, several works were produced to directly convert fibroblast into neurons (iN; Vierbuchen et al. 2010) and finally into functional DA neurons (iDA; Caiazzo et al. 2011).

In contrast, several problems are associated with iPSC generation such as genomic instability and chromosomal aberrations, raising interrogatives about their therapeutic use (Hussein et al. 2011; Pera 2011). Nevertheless, they constitute an invaluable tool to modelling several pathologies such as schizophrenia (Brennand et al. 2011), PD (Byers et al. 2012), Huntington's disease (Camnasio et al. 2012) and Alzheimer's disease (Yagi et al. 2011).

1.4.2 Neural stem cells

Neural stem cells (NSCs) are defined by their ability to proliferate, self-renew and to give rise to all the cell lineages of the brain, namely neurons, astrocytes and oligodendrocytes. These features are defined by the competence of NSC to undergo symmetric or asymmetric divisions depending on developmental stage. During early neurogenesis of the embryonic mouse brain, most NSC divisions are symmetric to expand the NSC pool and to establish a stable niche compartment. With the progression of neurogenesis, an increasing fraction of asymmetric divisions gives rise to progenitor cells, which migrate to their final positions (Götz and Huttner 2005; De Filippis and Binda 2012). Regarding the embryonic development, the term “neural stem cell” can identify a number of neuronal precursors, capable to originate both neurons and glia, with different features. First, in the embryonic ventricular zone *neuroepithelial* stem cells generate most of the neurons and glia in the brain, already at the start of neurogenesis these cells are gradually replaced by *radial glia*, as shown by fluorescent-activated cell sorting experiments (Malatesta et al. 2000). Radial glial cells show several astroglial properties and express markers known to the glial lineage, such as GFAP, however recent evidences raised the intriguing possibility that radial glia may also be stem cells/progenitors during embryonic development (Alvarez-Buylla et al. 2001). The stem cell/progenitor function of radial glia and astrocytes strongly suggests that stem cells are found within the neuroepithelial→radial glia→astrocyte lineage (Doetsch 2003b).

Also adult NSC are thought, in turn, to retain a glial identity, as showed by literature data showing that cells with characteristics and markers of embryonic radial glia are found only in the adult SVZ and SGZ (Doetsch et al. 1999; Kriegstein and Alvarez-Buylla 2009). In adult mammals, NSCs are mainly confined in the “neurogenic” areas, that are the subgranular zone (SGZ) in the dentate gyrus of the hippocampus and the subventricular zone (SVZ) of the lateral ventricles (Gage 2000). These areas are the niches that regulate NSC self-renewal, activation and differentiation (Doetsch 2003a). In the niches astrocytes serve as both neural stem cells and niche cell and "embryonic" molecular morphogens and signals persist in these niches and play critical roles for adult neurogenesis (Alvarez-Buylla and Lim 2004).

Many factors can regulate NSC homeostasis, such as environmental signals from the niche as well as cell-cell interactions and intrinsic factors involved in the regulation of the balance between multipotent/proliferative state and differentiation. Astrocytes provide rapid propagation of signaling within the stem cell niche. Furthermore, astrocytes themselves secrete factors that support neurogenesis *in vitro* (Lim and Alvarez-Buylla 1999). Also Noggin, produced by ependymal cells in the niche, is responsible to maintain the neurogenic potential of NSC by antagonizing *bone morphogenic proteins* (BMP) signaling, which instead promotes differentiation (Lim et al. 2000). On the other hand, *in vivo* and *in vitro* experiments have provided a strong role for EGF signaling in the induction and maintenance of the multipotency (Doetsch et al. 2002). Interestingly, oxygen plays a critical role in regulating the growth and differentiation state of NSC. *In vitro* and *in vivo* various studies show that low O₂ increases the hypoxia-inducible factor 1alpha (HIF-1 α), which promotes self-renewal and inhibits NSC differentiation or apoptosis while increased O₂ tension has opposite effects (Panchision 2009; De Filippis and Delia 2011).

Given their unique features, NSCs are considered to be the best candidate for replacement therapy in the treatment of a number of nervous system disorders. Indeed, transplantation of stem cells or their derivatives in animal models of neurodegenerative diseases can improve function by replacing the lost neurons and glial cells and by mediating remyelination, trophic actions, and modulation of

inflammation (Lindvall and Kokaia 2010). For example, transplantation of human neural progenitor cells genetically modified to secrete GDNF into the striatum and substantia nigra protected injured DA neurons (Behrstock et al. 2006). Similarly, NSC injected into substantia nigra and striatum of nonhuman primates mediated recovery, possibly by giving rise to a small number of cells with DA neuron phenotype (Redmond et al. 2007). Cell transplantation studies has given promising results also for *amyotrophic lateral sclerosis* (ALS; Xu et al. 2006; Xu et al. 2009), spinal cord injury (Cummings et al. 2005) and stroke (Kelly et al. 2004; Bacigaluppi et al. 2008; Bacigaluppi et al. 2009). However in many models of neurodegenerative diseases, on the long-term the “affected” brain environment may damage healthy engrafted neurons resulting in a exacerbation of the pathology, as showed by literature data (Li et al. 2008; Brundin et al. 2008; Desplats et al. 2009).

Strikingly, recent evidence has carved out a role for NSCs as immunomodulators. Indeed they are shown to be able to promote neuroprotection by maintaining undifferentiated features and exerting unexpected immune-like functions (Pluchino et al. 2005). Moreover, *toll-like receptors* (TLR), tightly associated with inflammatory response, are expressed by NSCs controlling their proliferation and differentiation properties. This suggests that TLR activation on NSCs regulates neurogenesis in response to injury and inflammation (Martino and Pluchino 2007). Finally, in experimental autoimmune encephalomyelitis (EAE) models, transplanted NSC control immune system activation through a bystander effect (Pluchino et al. 2009).

Pathological conditions can be associated to alterations in NSC homeostasis. Recent evidence suggest that the perturbation of molecular mechanisms that normally regulate the balance between proliferation and differentiation in NSC can be, at least in part, associated with the progression of malignant brain tumors (Verginelli et al. 2013; Modrek et al. 2014). The view that tumor stem cells underpin the development and/or maintenance of brain cancer has been confirmed by their identification in tumors of the central nervous system (Ignatova et al. 2002; Singh et al. 2003).

Other studies indicate the possibility that mutations of the *leucine-rich repeat kinase 2* (LRRK2), common in sporadic forms of PD, could be associated with dysfunctional NSC pools and might contribute to the hippocampal and SVZ-related age-dependent non-motor symptoms, including depression, anxiety and hyposmia (Liu et al. 2012).

1.4.3 Neurogenesis in the adult

At the begin of '900 the father of neurobiology Santiago Ramon y Cajal wrote: "*Once development was ended, the fonts of growth and regeneration of the axons and dendrites dried up irrevocably*", the so called central dogma of neurobiology that strengthened the idea that new neurons could not be formed and integrate in an adult brain (Colucci-D'Amato et al. 2006). The relative recent discovery of NSC in the brain along with the latest experiments on their function put an end to debate opening a totally new field of interests about neurogenesis in the adult, and its consequences. The first evidence of neurogenesis in the adult brain goes back to the 1960s when Joseph Altman showed that cells in the dentate gyrus of the hippocampus could incorporate radioactive thymidine (Altman 1962). Later, Kaplan and Hinds, using the electron microscopy coupled with autoradiography, demonstrated that the thymidine-labeled cells were neurons (Kaplan and Hinds 1977). In the 1980s, Nottebohm and his colleagues published a series of elegant studies showing that a substantial number of new neurons are produced in the song system of adult birds (Paton and Nottebohm 1984; Nottebohm 1989). However, the first evidence of a cell with "*stemness*" features in the adult brain came, for the first time, in 1992 when Reynolds and Weiss employed a serum-free culture system known as the neurosphere assay (NSA), to isolate and expand the putative stem cells from the adult brain, wherein most of the primary differentiated CNS cells harvested would not be able to survive (Reynolds and Weiss 1992).

As pointed out above, in adult mammals NSCs are confined to SGZ generating granular neurons, and the SVZ where new neuroblasts are continuously produced and migrate through the rostral migratory stream (RMS) to the olfactory bulb (Fig.8; Gage 2000; Curtis et al. 2007).

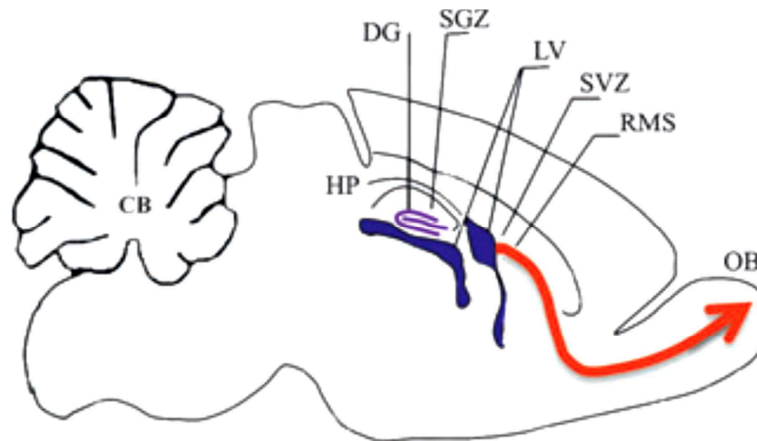


Figure 8. Neurogenic areas in the adult brain. The diagram represents a sagittal section of mouse brain showing the two canonical regions of adult neurogenesis: the subgranular zone (SGZ) of the dentate gyrus (DG, violet) in the hippocampal formation (HP), and the subventricular zone (SVZ) of the lateral ventricle (LV). The neural progenitors generated in SVZ will reach the olfactory bulbs (OB) through the rostral migratory stream (RMS, red).

In adult birds the new cells are generated from lateral ventricles and migrate to their final destinations, where they attained morphological and ultrastructural characteristics of neurons, extended axons into appropriate target regions, received synaptic input and were activated by auditory stimuli (Alvarez-Buylla et al. 1988).

The newborn neurons in the hippocampus have enhanced synaptic plasticity for a limited time after their differentiation which is critical for their role in mediating pattern separation in memory formation and cognition in rodents (Ge et al. 2007; Clelland et al. 2009; Deng et al. 2009).

A still opened question is whether neurogenesis is constrained only in the two canonical zones, SVZ and SGZ, or could occur also in other brain areas (Gould 2007). A seminal work by Gould and collaborators demonstrated that neuroblasts generated in the SVZ migrate in three different regions of the neocortex in adult macaque (Gould et al. 1999) while other brain areas are subjected to a “local” neurogenic potential following injuries or lesions, such as the rat visual cortex (Sirko

et al. 2009), the layer VI of the cortex (Magavi et al. 2000) and also the spinal cord meninges that harbor nestin- and doublecortin-positive cells, which are activated after injury and migrate into the spinal cord forming glial scars *in vivo* and can give rise to neurons and oligodendrocytes *in vitro* (Decimo et al. 2011). Strikingly, recent works provided evidences for neurogenesis in the adult mammalian *substantia nigra* where newly generated dopaminergic projection neurons derive from a population of actively dividing progenitor cells lining the cerebroventricular system in the midbrain (Lie et al. 2002). Although the number of neurons generated is orders of magnitude lower than in the hippocampus or the olfactory bulb, the estimated turnover rate implies, provided the rate is constant, that the entire population of SN neurons could be replaced within the life span of the mouse (Zhao et al. 2003).

What about humans? One of the first evidences of neurogenesis in the adult human hippocampus was provided by Eriksson *et al.* by studying bromodeoxyuridine (BrdU) incorporation in *post-mortem* human brains (Eriksson et al. 1998). Subsequent studies estimated the extent of adult neurogenesis in humans, quantifying the number of cells expressing the neuronal precursor marker *doublecortin* (DCX) in the subventricular zone, which gives rise to olfactory bulb neurons, and in the dentate gyrus of the hippocampus. These two regions contain a large number of neuroblasts shortly after birth that decreases sharply during the first postnatal year and then declines more moderately through childhood and adult life (Knoth et al. 2010; Sanai et al. 2011; Wang et al. 2011). By using an elegant cell birth dating technique, which consist in measuring the concentration of nuclear bomb test-derived ^{14}C in genomic DNA (Spalding et al. 2005), Spalding and co-workers recently demonstrated the generation of hippocampal cells in human brains estimating that 700 new neurons are added in each hippocampus per day, corresponding to an annual turnover of 1.75% of the neurons within the renewing fraction, with a modest decline during aging (Spalding et al. 2013). Using a similar approach, Ernst *et al.* were able to show that in adult humans new neurons, generated in the lateral ventricle wall of the brain, integrate in the striatum and that these are preferentially depleted in patients with Huntington's disease (Ernst et al. 2014).

It is now well established that adult neurogenesis can be increased by external stimuli, such as enriched environment (Kempermann et al. 1997), voluntary physical exercise (van Praag et al. 1999) as well as by dietary modulation (Park and Lee 2011). On the other hand, stress and depression can affect the generation of new neurons (Gould et al. 1998; Mirescu and Gould 2006; Castrén and Rantamäki 2010) while antidepressant treatment seems to have a positive effect (Malberg et al. 2000; Santarelli et al. 2003; Wang et al. 2008).

1.4.4 *In vitro* differentiation strategies to obtain DA neurons

Embryonic stem cells (ESC) and NSC offer a potentially simplified *in vitro* model system for the analysis of mDA maturation relative to the intact mammalian CNS. Furthermore, stem-cell-derived DA neurons may be of therapeutic benefit in cell replacement strategies for neurodegenerative disorders because of their potentially limitless supply. A number of studies have shown that ESC and NSC can generate cells with a mDA phenotype, and that the developmental program *in vitro* appears to recapitulate the temporal course of normal mDA maturation (Kim et al. 2003c; Barberi et al. 2003; Andersson et al. 2006b; Martinat et al. 2006; Young et al. 2010; Meyer et al. 2012).

The first *in vitro* studies on DA neurons differentiation and maturation were based on the reconstruction of the brain environment in which they develop. For instance, culturing mDA progenitors from embryonic brains in presence of their striatal target can induce a functional maturation of these cells (Prochiantz et al. 1979; di Porzio et al. 1980). In the same way, other protocols are based on the use of astrocyte conditioned media that could mediate a trophic and pro-survival action on differentiating progenitors as well as the use of striatal extracts that can enhance DA neurons maturation mediated by the presence of BDNF and GDNF (Daadi and Weiss 1999; Zhou et al. 2000).

Although all available evidence demonstrates that ESC have a greater capacity to produce dopaminergic neurons than NSC or other types of lineage-specific stem cells (Lee et al. 2000; Cho et al. 2008), there are some advantages in deriving DA neurons from NSC. Firstly, as a cell type already restricted to the neural lineage, NSC do not generate teratoma following transplantation. Secondly, NSC can be derived from fetal and adult brains, thus one could envisage the potential use of a patient's own NSC in cell therapy to avoid immune rejection (Gale and Li 2008). Beside the above cited neurosphere assay, several methods were developed to isolate and maintain neural stem/progenitors cells *in vitro* (Colucci-D'Amato et al. 1999; Volpicelli et al. 2004a; Conti et al. 2005; Pollard et al. 2006; Hook et al. 2011). One of the issues associated with these protocols is the maintenance of the self-renewal state by using several factors (Shi et al. 2008) to propagate a cell population that has to be as homogeneous as possible. For instance, it has been shown that TH-positive neurons were generated from bFGF-expanded NSCs, but only in early passages; these results suggest that primary TH-positive neurons may be carried over at the time of dissection or the expanded cells may not be NSC but constantly differentiating progenitor cells (Studer et al. 1998).

Basically, there are two way to differentiate NSC *in vitro*: by adding soluble extrinsic factors to the culture media or by ectopically introduce into NSC essential intrinsic factors such as key transcription factors.

One of the hallmarks of many *in vitro* differentiation protocols is the recapitulation of the principal stages of development: induction and specification phase, in which are principally used morphogens and mitogens, followed by a differentiation stage in which are used molecules and factors that promote DA differentiation and survival. Among inducers, the action of morphogens SHH and FGF8 was shown to be efficient to induce DA fate (Hynes et al. 1995; Volpicelli et al. 2004a; Volpicelli et al. 2004b). SHH signaling play a central role both in the proliferative phase of NSC and in the responsiveness of the latter to DA-inducing factors (Kim et al. 2003c; Martínez et al. 2013).

Wnt and TGF- β were also shown to have a strong role in the induction and specification of DA cells *in vitro* (Farkas et al. 2003; Roussa and Krieglstein 2004; Roussa et al. 2008; Rössler et al. 2010).

The other task of the differentiation protocols is to ensure the correct maturation and survival of DA cells by using differentiation and survival factors, such as BDNF (Engele 1998), GDNF (Consales et al. 2007), ascorbic acid (Volpicelli et al. 2004a), and *cyclic adenosine monophosphate* (cAMP; Michel and Agid 1996).

Other environmental conditions are shown to enhance DA differentiation, for instance low oxygen during bFGF-mediated expansion resulted in a significant increase in TH⁺ dopaminergic neurons as compared to high oxygen tension (Jensen et al. 2011). In the same way, depolarization induced by KCl treatment can increase DA differentiation (Volpicelli et al. 2007). In particular, recent works have shown that this treatment promotes DA neuron differentiation by opening chromatin structures surrounding DA related genes and by inhibiting the binding of co-repressors (He et al. 2011). Other studies take in account the action of molecules that are known to trigger pro-dopaminergic pathways, for instance it has been shown that the overexpression of the transmembrane molecule *B-cell lymphoma-extra-large* (Bcl-XL) not only exerts its known anti-apoptotic effect but also induces proneural and DA-related transcription factors, resulting in a high yield of DA neurons with the a phenotype resembling those of SN *pars compacta* (Park et al. 2006b; Courtois et al. 2010).

A similar approach may be taken into account to program dopaminergic competent NSC by exploiting transcription factors known to act in this specific neuronal lineage. Interestingly a great number of studies point on the above cited TF Nurr1, whose overexpression has been shown to efficiently induce DA neurons (Wagner et al. 1999; Kim et al. 2003a; Kim et al. 2003c). However, although Nurr1-induced cells are positive to DA markers, they show an immature phenotype (Park et al. 2006b; Park et al. 2012).

For this reason, other experiments suggest the co-expression of other TF such as Foxa2 (Lee et al. 2010) and Brn4 (Tan et al. 2012), while a number of articles highlight a possible cooperative mechanism with bHLH factors (Park et al. 2006a; Park et al. 2006b; Kim et al. 2007; Park et al. 2008). Other studies will clarify the nature and the mechanisms of these cooperation.

1.5 Aim of the project

As described in the preceding part, the development of dopaminergic neurons, at molecular level, is a highly concerted process in which extracellular signals and sequential activation of specific transcription factors (TFs) are integrated to define their physiological, morphological, and molecular phenotype.

A crucial task is to unveil the network of interactions and the hierarchy of molecular events underlying the acquisition of the dopaminergic function.

Previous data in the literature suggest that, among others, two TFs could cooperate to determine DA neurons identity: Nurr1 and Mash1.

Nurr1 is an orphan nuclear receptor member of the steroid-thyroid hormone receptor superfamily expressed in mDA post-mitotic progenitors starting from E10.5 and persists in the adult brain. Nurr1 is essential for mDA neurons development as its loss results in the agenesis of mDA neurons (Zetterström et al. 1996; Saucedo-Cardenas et al. 1998). Indeed Nurr1 is a direct regulator of a number of key DA specific genes such as TH (Kim et al. 2003b), DAT (Sacchetti et al. 2001), VMAT2 (Colebrooke et al. 2006).

Mash1 (Ascl1) is a bHLH transcription factor known as *proneural*. It is essential for neuronal specification in early developmental stages (Kageyama et al. 1997; Bertrand et al. 2002). It is expressed from early stages of embryonic development in different brain areas including midbrain where is induced by Otx2 at around E10 and last during the early post-mitotic phase (Vernay et al. 2005), however a role for Mash1 during later phases has not yet been described. Previous studies suggest that Mash1 might have a broad role in the subtype specification of different neuronal populations (Kim et al. 2008).

Interestingly, in developing midbrain, Mash1 expression overlaps with that of Nurr1 in a subset of Ahd2⁺ cells (Kele et al. 2006; Park et al. 2006a; Nelander et al. 2009). Similar studies underline the importance of Mash1 and Nurr1 in the reprogramming of somatic cells into dopaminergic neurons (Caiazzo et al. 2011).

I hypothesized that these two TFs may cooperate during the dopaminergic lineage commitment by controlling the switch from the specification phase to the post-mitotic differentiation fate, in order to trigger the molecular events leading to the correct development of fully differentiated midbrain DA neurons.

The aim of this project is thus to push Neural Stem Cells, obtained from embryonic brains with the neurosphere assay, to differentiate towards dopaminergic fate through the overexpression of Mash1 and Nurr1. The role of these TFs has been studied by characterizing differentiated cells from a morphological and functional point of view through the analysis of the DA markers TH, DAT, VMAT2 and Pitx3. In parallel I clarified the transcriptional regulation of genes of interest in the DA lineage, upon Mash1 and Nurr1 overexpression, using molecular biology approaches.

Altogether these data will shed light on the molecular and cellular events underlying the differentiation of DA neurons to enhance their generation *in vitro* and *in vivo* for possible future therapeutic purposes.

2. MATERIAL AND METHODS

2.1 Cell cultures

Materials for tissue culture, like multiwell plates, serological pipettes or pipette tips, were purchased from Corning, BD Biosciences or Nunc. All steps were carried out inside a laminar flow sterile hood (Jupiter) to avoid contamination. All the equipment was sprayed with Ethanol (70%) before use. Cells were incubated at 37°C with 5% CO₂ in a humidified incubator (Thermo Forma). Dissections were performed inside a horizontal flow hood (Hermes II).

2.1.1 Animals and dissections

Timed pregnant wild type or TH-GPF C57 BL-6 mice were sacrificed in accordance with Society for Neuroscience guidelines and Italian law. The embryonic age (E) was determined by considering the day of insemination (as confirmed by vaginal plug) as day E0. Embryos from day 12.5 (E12.5) of gestation were quickly removed and placed in phosphate buffered saline (PBS), without calcium and magnesium, and supplemented with 33mM glucose. The ventral midbrain or cortex was carefully dissected under a stereoscope in sterile conditions and processed for cell cultures. Tissues were pooled and triturated with a chemical and mechanical dissociation.

2.1.2 Neurospheres cultures

Single cells were obtained from embryonic cortex and midbrain as previously described (di Porzio et al. 1980). Briefly, the tissues were transferred into a 15 mL tube and treated for 30 min at 37°C with a solution containing papain (Worthington, 20 U/mL) dissolved in EBSS (Invitrogen) with the addition of cysteine (Sigma-Aldrich; 1 mM) and EDTA (Sigma-Aldrich; 1 mM) and 0,01% pancreatic DNase. After addition of 1 mg/mL of bovine serum albumin (Sigma-Aldrich) and 1 mg/mL ovomucoid (Sigma-Aldrich) and mechanical dissociation with a sterile pipette, the

cell suspension was centrifuged 5 min at 500 rpm, resuspended in plating medium and counted. For the viable count, cell suspension was diluted 1:10 with 0,1% trypan blue and loaded into a Burker's counting chamber slide. Cell concentration was determined on the basis of the total cell count, the dilution factor and the trypan blue exclusion.

Dissociated cells were plated in suspension at a density of 3×10^4 /mL in 75cm² tissue culture flasks (Corning). Cells were grown in DMEM/F12 with GlutamaxTM (Invitrogen) supplemented with N2 (Invitrogen), B27 without retinoic acid (Invitrogen), 100 U/ml Streptomycin, 100 µg/ml Penicillin (Sigma), bFGF (20ng/mL; Sigma) and EGF (20ng/mL; Sigma). After 3 days in culture (*days in vitro*, DIV) clonal aggregates, called neurospheres, were observed and after 5 DIV, as they reach $\approx 150\mu\text{m}$ diameter, were collected in a 15 mL tube, sedimented for 5 min at 200g and resuspended in 1 mL of TrypLE reagent (Life Technologies). Cells were incubated at 37°C for 4 min, mechanically dissociated using a wetted P1000 pipette tip, diluted 1:6 in DMEM/F12 and centrifuged for 5 min at 500g. Subsequently, the cells were resuspended in plating medium and counted as described above. For each passage, counted cells were plated at the same initial density.

2.1.3 Neurospheres differentiation into neurons and astrocytes

For the demonstration of the multipotency, cells were differentiated as previously described (Rietze and Reynolds 2006). Briefly, neurospheres were harvested, dissociated and counted as described above, and then single cells are plated at a density of $2,5 \times 10^5$ cells/cm² on glass coverslips for immunostaining analyses or 12-well plate for RNA extraction. Both coverslips and multiwells were previously coated with 15µg /ml poly-D-lysine (Sigma) for 1 hour at 37°C and washed three times with sterile H₂O. Cells were allowed to differentiate in DMEM/F12 supplemented with 1% FBS (Euroclone), 100 U/ml Streptomycin and 100 µg/ml Penicillin (Sigma). After 3 DIV half of the medium was replaced with fresh medium and at 6 DIV cells were fixed for immunostaining or harvested for RNA extraction.

To obtain individual cultures of neurons or astrocytes a different protocol were used according with Johe et al. (Johe et al. 1996). Neurospheres were collected, dissociated and plated on coated coverslips or 12-well plates at a density of 6×10^4 cells/cm². Cells were expanded in DMEM/F12 supplemented with N2 (Invitrogen), B27 (Invitrogen), 100 U/ml Streptomycin, 100 µg/ml Penicillin (Sigma) and bFGF (20ng/mL; Sigma-Aldrich) for 24-48h. Upon bFGF withdrawal, cells were allowed to differentiate for 6 days in presence of 10 ng/mL PDGF-BB (Sigma-Aldrich) to enhance neuronal differentiation or 10 ng/mL CNTF (Sigma-Aldrich) to push astroglial fate. Half of the medium was replaced every 3 DIV then cells were fixed for immunostaining analyses or processed for RNA extraction.

2.1.4 Generation of dopaminergic precursors from neurospheres

To properly differentiate neurosphere-derived cells, I adapted the protocol used in our lab for the expanded cultures (Volpicelli et al. 2004b). In brief, neurospheres, derived from cortex and midbrain, were collected, dissociated at single cells and counted as described above. Cells were plated at a density of 3×10^4 /cm² onto poly-D-lysine coated coverslips or multiwells. The protocol consists of a proliferative/inductive phase and a differentiation stage. For the first phase cells were allowed to proliferate under inductive conditions for 6 DIV in DMEM/F12 supplemented with N2 (Invitrogen), B27 without retinoic acid (Invitrogen), 100 U/ml Streptomycin, 100 µg/ml Penicillin (Sigma), bFGF (20 ng/mL; Sigma-Aldrich), N-terminal-SHH (50ng/ml) and FGF8 (10 ng/ml; Sigma). At DIV 3 half of the medium was replaced and inducible lentiviruses (see below) carrying the sequences for Mash1, Nurr1 or GFP (negative control) were added at respective dilutions of 1:10, 1:15 and 1:10. At DIV 6, for the switch to the differentiation phase, proliferative medium was replaced with DMEM/F12 supplemented with N2 (Invitrogen), B27 with retinoic acid (Invitrogen), 100 U/ml Streptomycin, 100 µg/ml Penicillin (Sigma), Ascorbic acid (200 µM; Sigma), 1mM dibutyryl cyclic adenosine 3', 5'-monophosphate (cAMP; Sigma), GDNF (10 ng/mL; Sigma).

The expression of transgenes was induced by the addition of Doxycycline (DOX) (4µg/ml; Clontech) to the medium. At DIV 12 cells were fixed or collected for further analyses.

2.1.5 A1 cells cultures and differentiation

Mes-c-myc-A1 (A1) cells were generated from a single clone of immortalized cells, by means of c-myc infection, from E11 mouse embryonic mesencephalon (Colucci-D'Amato et al. 1999). A1 were cultured in minimal essential medium MEM/F12 (Invitrogen) supplemented with 10% FBS (Euro clone) and 100 U/ml Streptomycin, 100 µg/ml Penicillin (Sigma). Before seeding the cells plate were previously coated with 15µg /ml poly-D-lysine (Sigma) for 1 hour at 37°C and washed three times with sterile H₂O.

For Mash1 and Nurr1 over-expressing lentiviruses infections, cells were plated at 5000/cm² density and infected 24 hours later. At the same time, insert transcription was induced by the addition of Doxycycline (DOX) (4 µg/ml; Clontech) to the culture medium.

To differentiate A1 cells, 48 hours after the infection, serum containing medium was replaced by MEM/F12 supplemented with N2 (Invitrogen), 1mM dibutyryl cyclic adenosine 3', 5'-monophosphate (cAMP; Sigma), N-terminal-SHH (50ng/ml), FGF8 (10 ng/ml; Sigma) and Ascorbic acid (200 µM Sigma). Medium was replaced once every three days. Cells were harvested after 6 days.

2.1.6 HeLa cells cultures

HeLa are an immortalized epithelial human cell line derived from cervical cancer cells (Scherer et al. 1953) . HeLa were maintained at 37°C with 5% CO₂ in a humidified incubator, in DMEM (Invitrogen) with 10% fetal bovine serum (FBS; Euroclone) and 100 U/ml Streptomycin (Sigma) and 100 µg/ml Penicillin (Sigma).

2.1.7 HEK 293T cells cultures

Human embryonic kidney 293T (HEK293T) cells were generated from human embryonic kidney cells obtained from a single apparently healthy fetus legally aborted. In the early 70s, these cells were genetically transformed with Adenovirus 5 DNA to obtain a stable cell line (Graham et al. 1977).

Cells were maintained at 37°C with 5% CO₂ in a humidified incubator, in DMEM (Invitrogen) with 10% fetal bovine serum (FBS; Euroclone) and 100 U/mL Streptomycin (Sigma), 100 µg/mL Penicillin (Sigma) and 25mM Hepes (Invitrogen).

2.2 Immunocytochemistry

After medium removal, cells were washed twice with PBS and then fixed with 4% paraformaldehyde (PFA; Sigma) in PBS at RT for 30min. Cells were permeabilized for 15 min with PBS-0.1% Triton-X-100 (Sigma). Later, fixed cells were incubated in a blocking solution with 5% BSA (Sigma) and 10% of normal goat serum (NGS;) in PBS at RT for 1h and then with the primary antibody (the dilution was specific for each antibody), usually overnight at 4°C or 2h at RT. Subsequently, cells were washed three times in PBST, incubated 45 min-1h at RT with the fluorescence-labelled secondary antibody (1:400; Alexa Fluor[®], Invitrogen). In the end, cells were washed again in PBS and counterstained with DAPI (1:1000; Invitrogen). A complete list of the used primary antibodies is reported in Table 1.

Table 1. List of used primary antibodies for immunocytochemical stainings

Antigen	Manufacturer	Host	Dilution
TH	Millipore	Rabbit	1:400
TH	Chemicon	Mouse	1:400
Mash1	BD Pharmingen	Mouse	1:400
Flag (M2)	Sigma-Aldrich	Mouse	1:1000
Tuj1	Covance	Mouse	1:500
GFAP	Sigma-Aldrich	Rabbit	1:400
Nestin	Chemicon	Mouse	1:1000
Sox2	Millipore	Rabbit	1:700

2.2.1 Microscopy and quantitative analyses

Images were acquired using a Leica DMI 6000 inverted microscope. In order to compare pictures from the same set of experiments, images were taken keeping the same settings on the acquisition software (Leica Application Suite AF). For cell counts, the number of positively stained cells was quantified by using a custom macro in the ImageJ software. Briefly, 8-bit images from each channel were generated and converted in a binary image, and then the area of the smallest cell was measured. Cells were counted with the “analyze particles” tool by setting the standard circularity and the minimal size value obtained from the previous measure. For neuronal cells counting, a similar approach was used but, to overcome the generation of counting artefacts due to the peculiar shape of the cells, an image resulting from the comparison between the analyzed channel and the DAPI channel was generated with the “AND” operator in the Image calculator tool.

2.3 Molecular biology methods

2.3.1 Lentiviral vectors construction and transduction

Last generation lentiviruses have been engineered in order to contain less than 10% of the original viral genome. They express only sequences needed for reverse transcription and integration of the gene expression cassette into the host genome. To further increase the security level, these genes are expressed on three different vectors in a way to minimize the risk for a casual recombination event able to restore virus capacity to autoreplicate. The Psi (ψ) sequence, responsible for the viral genome packaging, is localized exclusively on the transfer vector containing the exogenous gene, to further minimize the possibility to generate functional autoreplicant viruses. The expression of the exogenous gene was under control of a Doxycycline-inducible promoter. The used vectors for the lentiviral particles production were: a Gag/Pol-containing pMDL vector, a pRev vector, a pVSV-G vector and the transgene-containing transfer vector Tet-O-Fuw. Moreover, a prtTA vector, expressing for the

reverse tetracycline transactivator (rtTA) protein, was necessary, in conjunction again with the pMDL, pRev and the pVSV-G, to produce lentiviruses to combine in the infections with the Tet-O-Fuw-containing viruses in order to allow the Doxycycline induced expression (rtTA vector was supplied by Massimiliano Caiazzo, PhD; Istituto San Raffele, Milan).

For all these vectors Gigapreps were made using the EndoFree Plasmid Gigaprep (Qiagen) following the manufacturer instructions. For the viral particles production, 8.2×10^6 HEK293T cells were plated in 150mm x 25 mm dishes in DMEM (Invitrogen) supplied with 10%FBS (Euroclone), 25mM Hepes (Invitrogen), 100U/ml Streptomycin (Sigma) and 100µg/mL Penicillin (Sigma). 24 hours later an 80% confluence is generally obtained. Medium was replaced by ISCOVE medium (Invitrogen), 10% FBS, 100U/ml Streptomycin and 100µg/ml Penicillin. 3 hours later a 4 vectors co-transfection was made following the calcium phosphate transfection protocol.

In details, for each dish, a solution was prepared containing 270mM CaCl₂, 6,25µg pRev, 9µg pVSVG, 14,6µg pMDL and 32µg if insert containing transfer vector. After 5 min incubation at room temperature, drop-by-drop, in low agitation, a 2xHBS pH 6,95 (NaCl 280mM, Na₂HPO₄ 1,5mM, HEPES 50mM) solution was added to the vectors mix. A 15 min room temperature incubation followed. Later, the entire solution was spread, drop by drop, in the culture medium and 293T cells were incubated at 37°C and 5%CO₂. 12 hours later, medium was refreshed and reduced in its amount. 30 hours later, medium was harvested, filtered (0.22µm filters) and the ultra-centrifuged at 19400 rpm at 20°C for 2 hours (Optima L90K; Beckman Coulter). Lentiviruses-containing pellet was resuspended in PBS and stored at -80°C. Lentiviruses infectivity and the “maximum tolerated dose” were empirically determined through sequential infections on HEK 293T cells with increasing amounts of virus.

2.3.2 RNA extraction and reverse transcription

RNA was extracted from cells using the TRI-REAGENT (Sigma) classic method as described by Chomczynski and Sacchi (Chomczynski and Sacchi 1987). RNA was treated with DNAase (Ambion) to eliminate possible DNA contaminations. The yield and the integrity of RNA were determined by spectrophotometric measurements and by agarose gel electrophoresis, respectively. For the reverse transcription 2µg of RNA were incubated for 5 min at 65°C with 6 µM random hexanucleotides as primers (New England Biolabs) in presence of 500 µM dNTP. Reverse transcription is performed with 200U of Moloney's murine leukemia virus reverse transcriptase (M-MLV, New England Biolabs) in an appropriate buffer (50mM Tris-HCl pH 8,3, 3mM MgCl₂, 75mM KCl, 10mM DTT) supplemented with 40U of RNase inhibitor (New England Biolabs). Samples were incubated in a thermal cycler (Celbio) at 25°C for 10 min, 37°C for 50 min and finally at 70°C for 15 min in order to denature RNA-cDNA hybrids. cDNA is diluted 1:5 in ddH₂O prior to use.

2.3.3 Quantitative PCR

Analyses were carried out on triplicate samples for each experiment and they were processed separately. RT-PCR was performed on 4µl of previously diluted cDNA (1:5) template using the Power Sybr Green Master mix (Applied Biosystem), in presence of 0.5uM specific oligos. Synthetic oligonucleotides were synthesized by Primm. The experiments were performed on a 7900 HT Fast Real time PCR system (Applied Biosystem) with a thermal profile consisted of 2 min at 50°C, 10 min at 95°C and 40 cycles of 15 sec at 95°C and 1 min at 60°C. All PCR data were normalized to the average of the reference gene *hypoxanthine-guanine phosphoribosyltransferase* (HPRT). All primers were designed using the Oligo7 software and are listed in Table2.

Table 2. List of gene specific primers for qPCR analyses

Gene	Forward primer	Reverse primer
DAT	TCTGGGTATCGACAGTGCCA	GCAGCTGGAACATCGACAA
DBH	GACCGGCTACTGCACAGACAA	GAGAGGCAAAGATGTGGATTCC
Foxa2	ACGAGCCATCCGACTGGAG	GGCGTTCATGTTGCTCACG
GAD2	AGGGTTACTGATGTCCCGGAA	ATGTTGGAGATGGCACCACC
GFAP	GAGGGACAACCTTTGCACAGGA	CCAGCCTCAGGTTGGTTTCAT
HPRT	TGCCCTTGACTATAATGAGTACTTCAG	TTGGCTTTTCCAGTTTCACTAATG
Lmx1a	AACCAGCGAGCCAAGATGAA	TGGGTGTTCTGTTGGTCCTGT
Mash1	GCCAACAAGAAGATGAGCAAG	CGTCGTGCTCGTCCAG
Nestin	AGCAACTGGCACACCTCAAGA	CTCAGCCTCCAGCAGAGTCC
Ng2	ATCTGGAGCCGCGTAGGAT	CATCAGTACCTCCTCTTCCTCCTTC
Nurr1	GTGCCTAGCTGTTGGGATGG	GTAAACGACCTCTCCGGCC
OCT 3/4	AACCAACTCCCGAGGAGTCC	CAGCAGCTTGGCAAACCTGTTC
Olig2	GGAGGAGATGAAGCGACTGGT	GAGGCGCCGAGTGTGC
Pitx3	GACGCAGGCACTCCACACC	TTCTCCGAGTCACTGTGCTC
PNMT	GTGTATAGTCAGCATGCCTGCC	CGAAGCTGGCGTTCTTTCTC
Sox1	GGAAAACCCCAAGATGCACA	CCTCGGACATGACCTTCCACT
Sox2	AGGGCTGGACTGCGAACTG	TTTGCACCCCTCCCAATTC
Sox3	GCACATGAAGGAGTACCCGG	TGTCCTTCTTGAGCAGCGTCT
TH	CTCACCTATGCACTCACCCGA	GGTCAGCCAACATGGGTACG
TPH1	ACAACATCCCGCAACTGGAG	CACAGGACGGATGGAAAACC
Vimentin	CGCCCTCATTCCCTTGT	GCGGTAGGAGGACGAGGACACA
Vmat2	TTGCTCATCTGTGGCTGGG	TGGCGTTACCCCTCTCTTCAT

2.4 Chromatin Immunoprecipitation

For chromatin isolation, midbrains were dissected from at least n.20 E12.5 mice embryos in ice cold PBS. The tissue was then weighted, homogenized using a glass potter and fixed in 1% formaldehyde in PBS at RT for 15 min to ensure crosslinking between proteins and DNA. Reaction was quenched with 125 mM glycine (Sigma) and tissue was washed three times with PBS supplemented with protease inhibitors (PI; Roche).

Pellets were resuspended in 1mL of lysis buffer (1%SDS, 50mM Tris-Hcl pH8, 10mM EDTA) supplemented with PI and incubated in ice for 10 min. Subsequently 0,5 mL of glass beads (Sigma) were added and tissue was incubated for additional 10 min in ice then sonicated to shear chromatin. Sonication was optimized to ensure a 200-500 bp average size. For this purpose a Soniprep150 disruptor (Sanyo) was used for 12-15 cycles 30''on/30''off with amplitude set to 11. After a 15 min centrifuge at 13.000 rpm to remove cell debris, DNA concentration was measured to the spectrophotometer. Usually a 0,5-1µg/µL-concentration range is considered acceptable. To verify the shear size, 50 µL of chromatin were decrosslinked at 65°C for 30 min with the addition of 3 µL of proteinase K (50mg/mL; Sigma), DNA was extracted with phenol/chloroform and loaded on a 1,5% agarose gel for a 30 min run at 150V. The result should display a DNA smear with a major concentration between 200bp and 500bp.

For the IP, up to 60 µg of chromatin were precleared with 50µL of sheep anti-mouse IgG-conjugated Dynabeads® (Invitrogen) for 1,5h at 4°C in rotation, then immunoprecipitated in IP buffer (0,2M HEPES pH 8, 0,2M NaCl, 0,02 M EDTA, 0,1% Deoxycholate Sodium, 1% Triton-X-100, 1mg/mL BSA and protease inhibitors) with 5 µg of anti-Mash1 (BD Pharmingen) or generic mouse IgG (Sigma) at 4°C ON in rotation. 50µL of input chromatin were processed separately as 5% input chromatin. The day after, the samples were incubated for 2h at 4°C in rotation with 50µL of Dynabeads® carrying the secondary antibody, to pull down the Mash1-enriched chromatin. Subsequently, the magnetic beads, which are bounded to immunoprecipitated chromatin, were captured and washed 8 times with a modified RIPA buffer (50mM HEPES pH 7.6, 1mM EDTA, 1% NP-40, 0,7% Deoxycholate Sodium) containing 0,5M LiCl. Chromatin was eluted 10 min at 65°C with a buffer containing 10mM Tris pH 8 and 1% SDS, then beads were removed. The same procedure was applied to input chromatin. To remove proteins, the IP chromatin were treated with 3µL of proteinase K (50mg/mL; Sigma) and 11µL of 5M NaCl at 65°C ON. DNA is isolated with 1 volume of phenol/chloroform/isoamyl alcohol (25:24:1; Euroclone) and precipitated with 5µL of glycogen, 50µL of 3M Sodium Acetate

pH5.2 and 900 μ L of isopropanol. DNA is therefore resuspended in 100-200 μ L of sterile ddH₂O.

For the analysis of the enrichment, 5 μ L of DNA were analyzed with RT-PCR as described above. The primers for the putative binding site, the positive control sequence and the negative control sequence were designed with Oligo7 software to with an average size of 20-24nt, a median T_m of 58-60°C and a 40-70% GC content. All used primers are listed in Table3. The input C_t value adjusted for the input percentage (Input C_t + Log₂5) was subtracted from all C_t values to normalize. ChIP efficiency was calculated as the ratio of precipitated sequence over total amount of sequence in the input chromatin. The data were plotted as means of at least two independent ChIP assays and three independent amplifications; error bars represent standard deviations.

Table 3. List of used primers for ChIP analysis

Binding site	Forward primer	Reverse primer
TH1	GCTGCCCAATTATCCCTAAG	GTAGGCACAGAATATGTACTAGA
TH2	GACCTCAAGTGCACCTCTTC	CCTCAGCTCCCAACACTTCT
TH3	AGGTGCTGGGAATAAGGTGG	TGTCTTAGGGAAGGGCTACA
NBRE	TGCCGAAAGGTTAAATCCACA	CCTGGCGTTTGCTCAGCTGG
DII1	GCGTGGCTGTCATTAAGG	GGTGCTGTCTGCATTACC

2.5 Western Blotting

To verify the enrichment of Mash1, 15 μ L of IP samples were decrosslinked at 65°C ON and protein concentrations were measured with Bradford assay.

Proteins were separated on 12% SDS-polyacrilamide gel and transferred to PVDF membranes (Millipore). Filters were probed for 2h at room temperature or overnight at 4°C with the anti-Mash1 antibody (1:500; BD Pharmingen). After washing, immunoblots were incubated with anti-mouse IgG antibodies (Amersham, 1:10000) and the reaction detected with the ECL plus procedure (Amersham).

2.6 Luciferase assays

HeLa cells or A1 cells were plated in 48-well plates (BD Biosciences) at a density of 40.000 cell/well. For the promoter analysis 0,4µg of the pGL3-NBRE plasmid and 0,4µg of 3xFLAG-Nurr1 and pcDNA3-Mash1 plasmids were co-transfected, using Lipofectamine 2000® (Invitrogen). I used a Renilla luciferase vector carrying the Simian vacuolating virus 40 promoter (pRL-SV40) as an internal control vector. For the dual (firefly and Renilla) luciferase assays, cells were extracted with passive lysis buffer (Promega). 10µL of cell extract were used for firefly and Renilla luciferase assays using the Dual-Luciferase™ Reporter Assay System (Promega) and the ratio of luminescence signals from the reaction mediated by the firefly luciferase reporter to those from the reaction mediated by the Renilla luciferase was calculated. For each reporter the luciferase activity of the empty pGL3 vector was subtracted. The data were performed in triplicate.

2.7 Bioinformatics analysis

To analyze TH promoter, an 8101bp sequence was retrieved on the NCBI database (<http://www.ncbi.nlm.nih.gov/nucleotide/>; ID: JN963248.1).

This sequence was inserted into Regulatory Sequence Analysis Tool (RSAT; <http://rsat.ulb.ac.be/rsat/>; Turatsinze et al. 2008) setting a position weight matrix (PWM) retrieved on Jaspar core database (<http://jaspardev.genereg.net/>; Mathelier et al. 2014). This matrix, Ascl2_1 (ID PB0003.1), encodes for a sequence CAGCTG in accordance with Castro et al. (Castro and Guillemot 2011). The resulting sequences have been ordered by increasing p-values and position weight scores and the more significant were chosen.

2.8 Statistical analyses

For all experiments, analysis of variance was carried out, followed by post hoc comparison (ANOVA, Bonferroni-test). A value of $p \leq 0.05$ was considered significant. Data were expressed as mean \pm SE. At least three independent replicates were used for RT-PCR and luciferase assays, and at least two replicates were used for cell counts and ChIP experiments, with data expressed as mean \pm SD.

3. RESULTS

3.1 Neural stem cells isolation, culture and characterization

One of the difficulties in identifying and studying stem cells is their poorly defined physical nature, which affects our ability to directly measure their presence and to monitor their activity. This problem has been overcome by a functional characterization rather than a physical or morphological observation.

In order to obtain an *in vitro* model system to study the differentiation of mDA neurons, neural progenitor/stem cells have been isolated from midbrains of embryonic day 12.5 (E12.5) mouse embryos. Embryonic cortex derived cells were also used as a comparison to evaluate the influence of the regional commitment on the specification of the neuronal subtype. However as described below, also these cells are able, under appropriate conditions, to generate TH positive neurons. The choice of the embryonic stage was driven by practical and theoretical reasons: TH⁺ cells, considered in the midbrain precursors of DA neurons, appear in mouse around E9. At E10-E11 their distribution is similar to that of cell clusters of A9 and A10 areas in the adult mouse while only at E13 these appear as separated areas (Di Porzio et al. 1990). Nevertheless cells obtained at E13 appear being more responsive to differentiation factors than those from E11 (*data not shown*). Thus I choose E12.5 as the stage with the better balance between precursors yield and responsiveness to differentiation factors. Cells were derived both by wild type (WT) and TH-GFP C57 BL/6 mice, a transgenic mouse line carrying the fluorescent protein EGFP under the control of TH promoter (Sawamoto et al. 2001).

Initially, to characterize the isolated cells, I performed a functional assay to assess their ability to proliferate, self-renew and to give rise to neurons, astrocytes and oligodendrocytes. The neurosphere assay takes account of these three characteristics.

3.1.1 Neurosphere-derived cells are able to self-renew

Cells derived from embryonic tissue were plated at low density (≈ 30.000 cells/mL) in a serum-free culture medium containing the mitogens bFGF and EGF. In these conditions they undergo cell division giving rise to floating aggregates, deriving from a single cell, called neurospheres. These aggregates show a characteristic 3-D structure with small protrusions at the edges and after 5-7 days *in vitro* (DIV), reach a ≈ 150 μm diameter, feature that demonstrate the ability of isolated cells to proliferate (Fig.9).

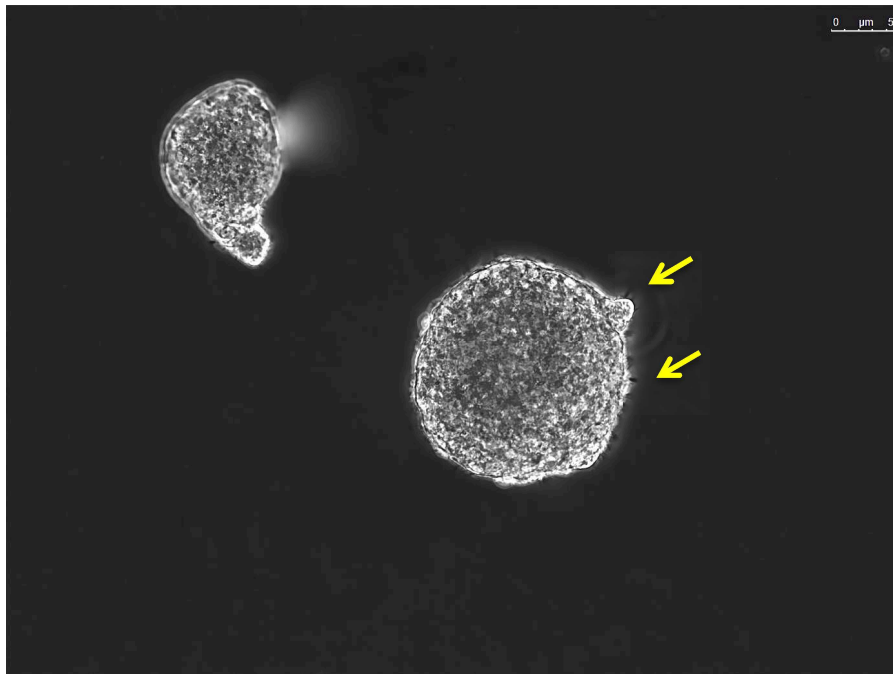


Figure 9. Cortex-derived neurospheres at 5 DIV. After 5 DIV clonal aggregates reach ≈ 150 μm diameter and show a characteristic structure. Arrows indicate characteristic small protrusions.

Once the spheres reached the appropriate dimensions, they were collected, dissociated and re-plated at the same initial density to monitor the formation of secondary spheres. At every passage, cells were counted to assess the ability to proliferate and self-renew. As shown in Fig.10, neurospheres derived from cortex have shown a 7,21-fold increase at every passage versus a 3,44-fold increase of midbrain-derived cells, a feature suggesting a lower proliferative capacity of the latter.

Nonetheless, the ability of the cells to proliferate at every passage and reform spheres with no significant decrease of the final cell density demonstrates the clear ability of both cell types to self-renew (Fig.10).

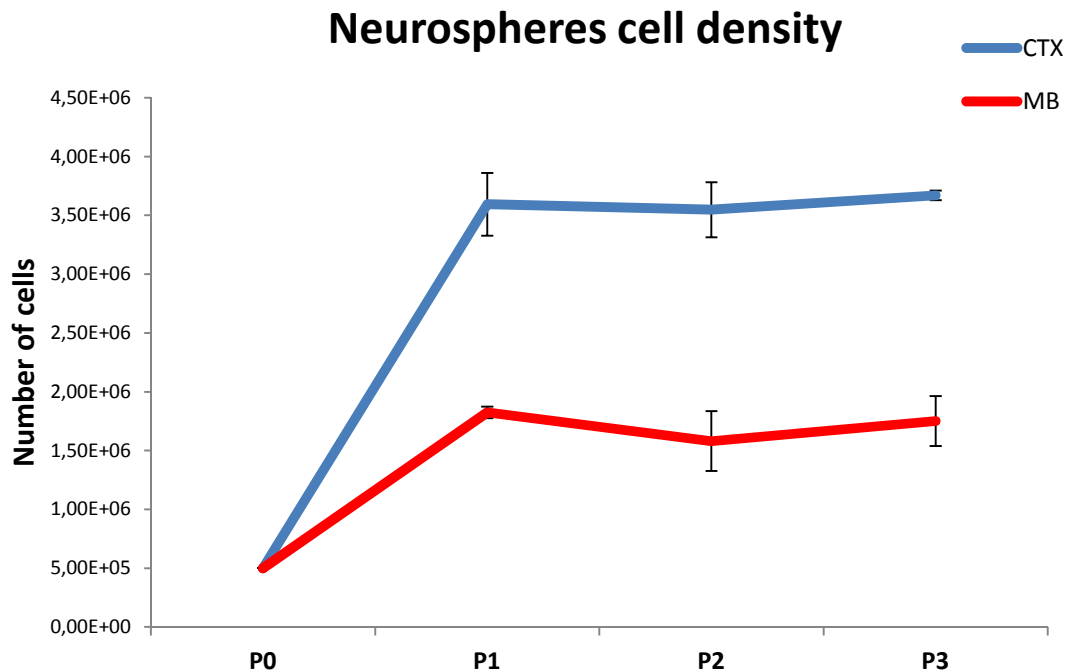


Figure 10. Neurosphere self-renewal assessment. The chart shows the trend of neural stem cells derived from cortex (CTX) and midbrain (MB) to proliferate and to maintain the same density during passages. Midbrain-derived cells (red line) show a minor propensity to proliferate as compared with cortex-derived cells (blue line).

3.1.2 Proliferating neurosphere-derived cells express progenitor markers

To evaluate the phenotypic stability of the cultures, I checked, during passages, for the expression of specific genes known as markers for neuronal progenitor and mature neurons. Several markers are known to be expressed in NSC, such as *Nestin* (Alvarez-Buylla et al. 2001; Campos 2004), *Sox-2* (Graham et al. 2003) and *Glial Fibrillary Acidic Protein* (GFAP), a protein expressed principally by astrocytes but also by NSCs (Doetsch 2003a; Doetsch 2003b). In my experiments, *Nestin*, *Sox-2* and *GFAP* were chosen as indicators of the immature phenotype, while β -*III tubulin* (Tuj1) was selected as marker of post-mitotic neurons. These markers allowed monitoring the NSCs in culture to ensure that they maintained a low degree of differentiation during passages.

Immunostaining experiments have shown that Nestin and Sox2 as well as GFAP were consistently expressed (Fig. 10-A). Notably Nestin and Sox2 transcripts were highly expressed during passages although appear to fluctuate. In particular, Sox2 seems to rapidly increase after the first plating (Fig.10-B). This is consistent with the hypothesis that only neural progenitor cells are able to amplify under the effect of mitogens. Undeniably, Tuj1 levels rapidly decrease after the first plating and maintain very low levels during passages (Fig. 10-B) in agreement with the previous hypothesis. The same trend in all markers was observed in both midbrain- and cortex-derived cultures, suggesting that NSC, isolated from either areas show similar features despite the different regional identity.

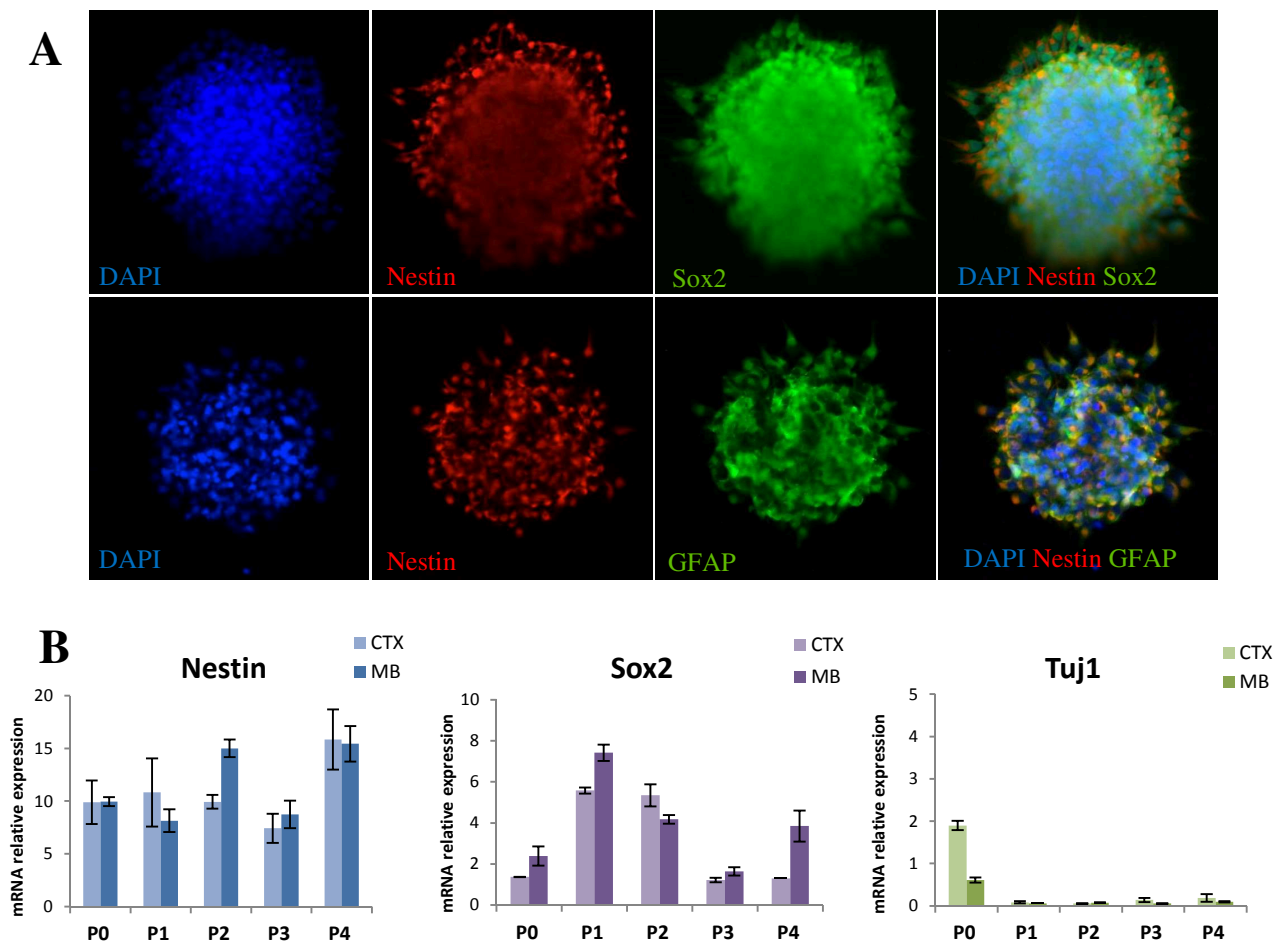


Figure 11. Neurospheres express progenitor markers. Markers of immature proliferating cells, such as Nestin, Sox2 and GFAP (A) appear to be regularly expressed in whole neurospheres. In (B) it can be observed that the expression of Nestin and Sox2 is stable through the passages while it can be noted the decrease of the mature neurons marker Tuj1 just after the first plating.

3.1.3 Neurosphere-derived cells are multipotent

As pointed out above, the proof for the multipotency of neurosphere-derived cells is the ability of the latter to form neurons, astrocytes and oligodendrocytes when allowed to differentiate. Neural progenitors proliferate in culture under the effect of mitogen factors such as bFGF and EFG, but they start to differentiate upon mitogens withdrawal and addition of 1% serum (Rietze and Reynolds 2006).

Thus, using semi-quantitative PCR and immunostaining, I evaluated, upon differentiation of the cells, the expression of Tuj1, specific for neuronal lineage, the astrocytic marker GFAP and the bHLH factor Olig2, specifically expressed in oligodendrocytes. Both cortex- and midbrain-derived cells are able to differentiate in the three lineages, as shown by immunostaining and gene expression analysis for the indicated markers (Fig.12). In particular, differentiated neurospheres from both areas show a similar percentage of neurons (3%), astrocytes (62%) and oligodendrocytes (1,3%). This result is consistent with previous works (Reynolds and Weiss 1992) showing the prevalence of astrocytic lineage in differentiated cultures.

A different approach in differentiating neurospheres is to induce a specific phenotype independently from the others. The treatment with specific growth factors can drive the progenitor cells towards a specific fate (Johe et al. 1996), in particular *Platelet-derived growth factor* (PDGF) is able to induce a neuronal phenotype, *Ciliary neurotrophic factor* (CNTF) is involved in the generations of astrocytes and *Triiodothyronine* (also known as T3) induces the formation of oligodendrocytes.

I therefore treated midbrain and cortex-derived cells with PDGF or CNTF to increase respectively neuronal and astroglial fate and analyzed expression of markers.

Subsequently, I evaluated mRNA and protein expression of neuronal, astroglial and oligodendroglial markers. Analysis for neuronal markers showed that PDGF selectively induced neuronal fate leading to a strong increase in Tuj1 expression both in immunolabeled cells and in Real Time PCR experiments (Fig.13-A and 13-B). In the same way CNTF was efficient in inducing astroglial lineage differentiation, however it has a positive effect also on neurons generation.

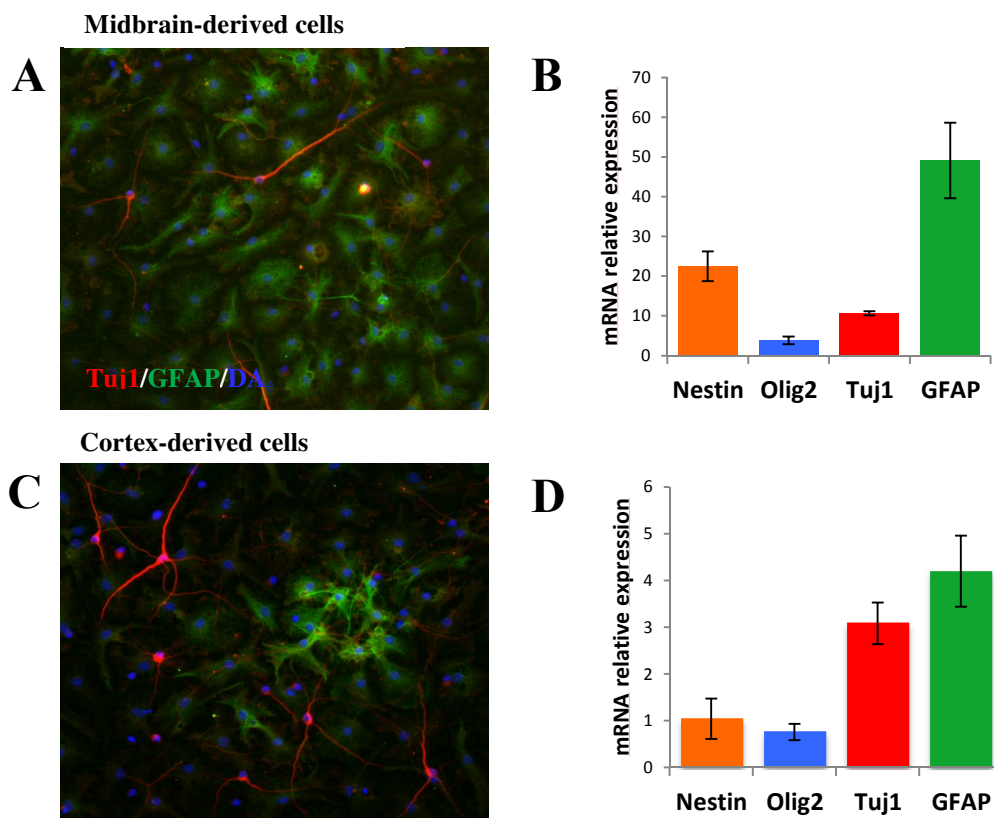


Figure 12. Neurosphere multipotent differentiation. Representative images of neurosphere-derived cells differentiated upon withdrawal of mitogens and serum addition. On the left are showed immunostainings for neuronal marker Tuj1 (red) and the astroglial marker GFAP (green). On the right side are represented relative mRNA amounts of lineage markers compared with the expression of progenitors marker Nestin. Midbrain-derived cells are showed in **A** and **B**, cortex-derived cells are showed in **C** and **D**.

As shown in Fig.13, PDGF treatment led to a higher differentiation of Tuj1⁺ cells with very few or no GFAP⁺ cells. On the other hand, in CNTF treated cultures I observed a strong increase in GFAP⁺ cells. Interestingly the massive presence of astrocytes could exert a trophic effect on neurons, also under these culture conditions, and favors a little neuronal differentiation, since neurons are present, albeit in small numbers, and survive as well.

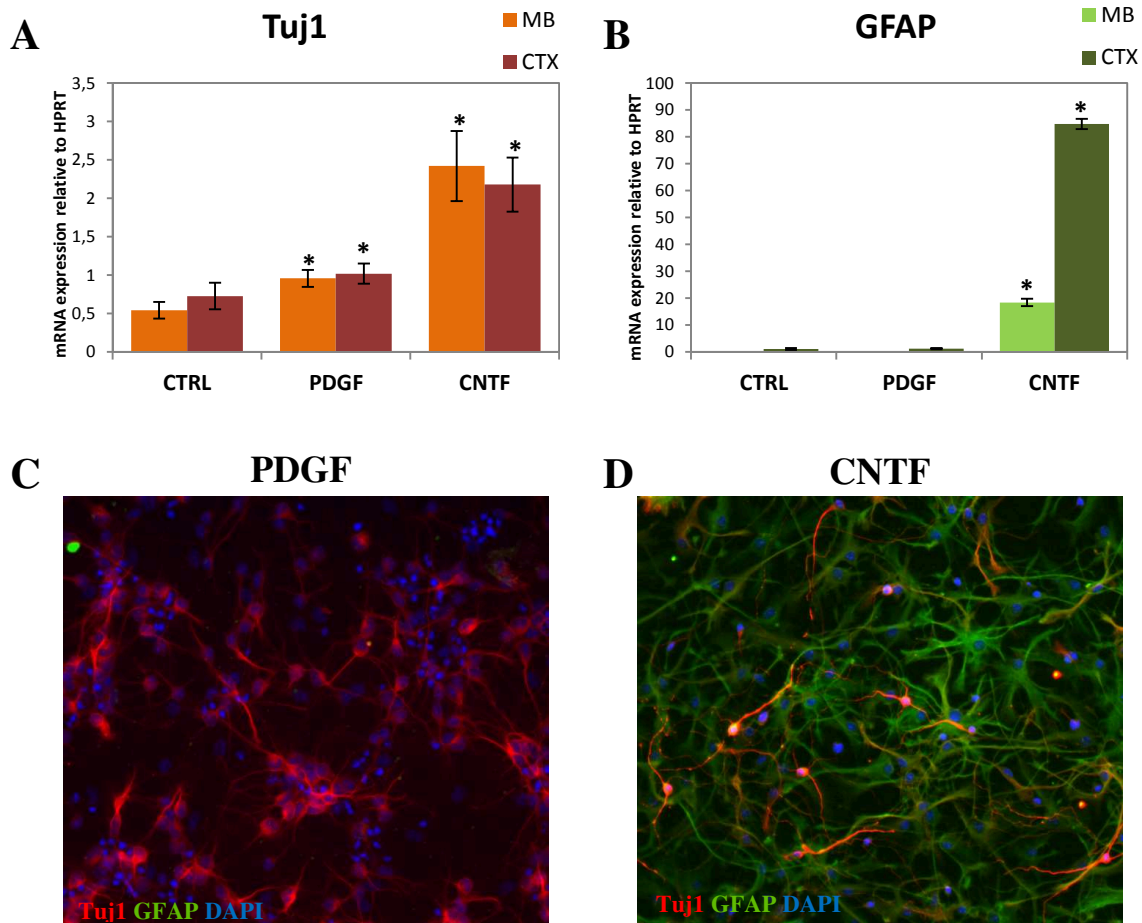


Figure 13. PDGF and CNTF treatment on neurosphere-derived cells. In A and B are represented mRNA levels of neuronal marker Tuj1 and astroglial marker GFAP following the treatment with PDGF or CNTF compared with an untreated control. Note that CNTF treatment results in an increase of neuronal types due to trophic action of astrocytes on the latter. In C and D Immunostaining for the same markers confirm the gene expression analysis as PDGF treatment results in the generation of only neurons (Tuj1⁺) while CNTF treatment increase the generation of astrocytes (GFAP⁺) with several neurons.

3.2 Differentiation of neural stem cells into dopaminergic neurons

To elucidate the molecular events underlying the differentiation of dopaminergic neurons under these experimental conditions, I established an *in vitro* paradigm that recapitulates, at least in part, the main molecular events occurring *in vivo*. First, neurosphere-derived cells are allowed to proliferate while exposed to the effect of known DA inducers in order to obtain proliferating DA precursors; then, upon mitogen withdrawal, cells are exposed to a number of factors that ensure DA survival and maturation. This basic protocol has been used as a platform to test the effect of Mash1 and Nurr1 overexpression (Fig.14).

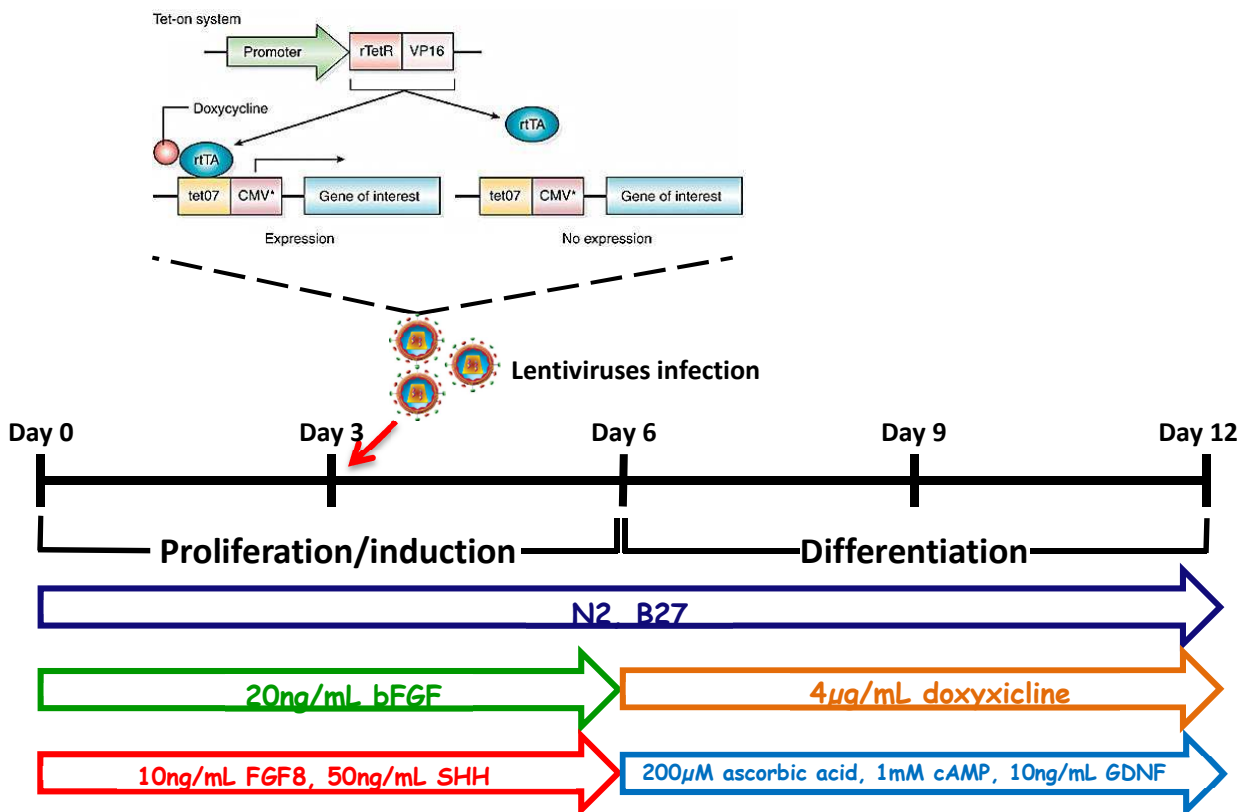


Figure 14. DA differentiation protocol. The scheme represents the protocol that consists of an induction and proliferation phase driven by the action of the mitogen bFGF and of the morphogens SHH and FGF8. During this phase TFs were expressed using inducible lentiviral vectors. Transgene expression is achieved by the addition of doxycycline in the second phase, in which the differentiation of DA precursors is achieved through the action of AA, GDNF and cAMP which have the task of ensuring the survival and maturation of post-mitotic DA neurons.

3.2.1 Induction of the dopaminergic phenotype by SHH and FGF8 treatment

To obtain dopaminergic neurons from NSC derived from embryonic brains, cells were exposed to the actions of morphogen factors SHH and FGF8. In the first part of the protocol, cells are proliferating under the effect of bFGF and exposed to 50 ng/mL SHH and 10ng/mL FGF8. This phase is necessary for the induction of dopaminergic fate. The second stage is characterized by the withdrawal of the mitogen and the addition to the culture medium of Ascorbic Acid and GDNF, two factors that improve survival. In this way cells stop to proliferate and start to differentiate. However this protocol, by itself, has a moderate efficiency in promoting DA differentiations. As shown in Fig.15, the relative quantification of mRNA expression shows only a 2-fold increment of TH.

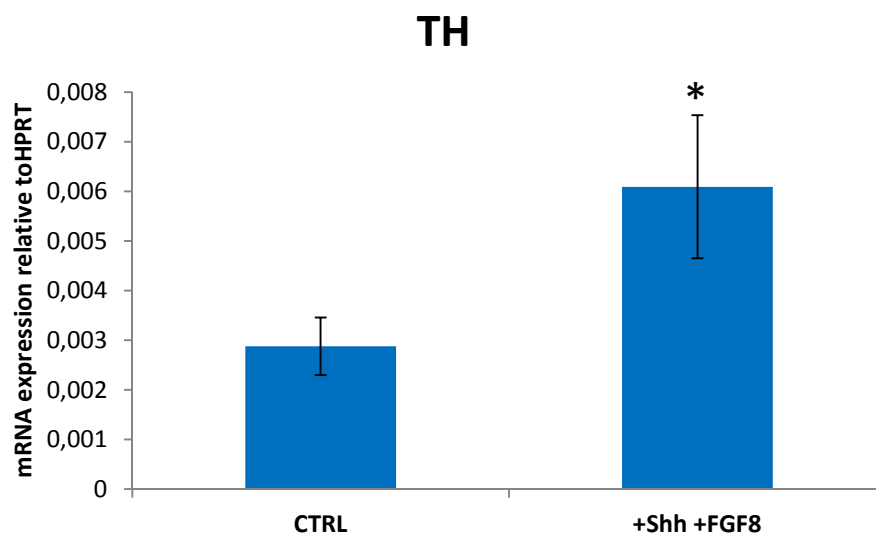


Figure 15. Relative expression of tyrosine hydroxylase after SHH and FGF8 treatment: addition to the culture medium of the morphogens elicits a 2-fold increment in TH expression

3.2.2 Overexpression of Mash1 and Nurr1 in neural progenitors enhance Tyrosine Hydroxylase gene expression

To study the effect of Mash1 and Nurr1 on the differentiation of DA neurons, I prepared inducible lentiviral vectors carrying their sequences. The use of inducible lentiviral vectors allows an efficient transduction of exogenous genes combined with the possibility to control gene expression by adding doxycycline to the medium.

Cells dissociated from midbrain and cortex-derived neurospheres were harvested from passage 2-4 cultures, plated at low density and allowed to proliferate for 6 days in presence of SHH and FGF8. During this stage Mash1, Nurr1, both the genes and an empty control vector were transduced to the cells. Their expression was induced only during the differentiation stage by adding doxycycline. Following the withdrawal of the mitogens and the induction of the exogenous genes, cells were allowed to differentiate for 6-8 days.

As expected the semi-quantitative PCR analysis shows a great increase in TH mRNA expression following Nurr1 infection. Infection with Mash1 alone resulted in a 2-fold increase (compared with the control) but interestingly, co-infection of Mash1 and Nurr1 resulted in a further 3-fold increase when compared with Nurr1 alone (Fig.16).

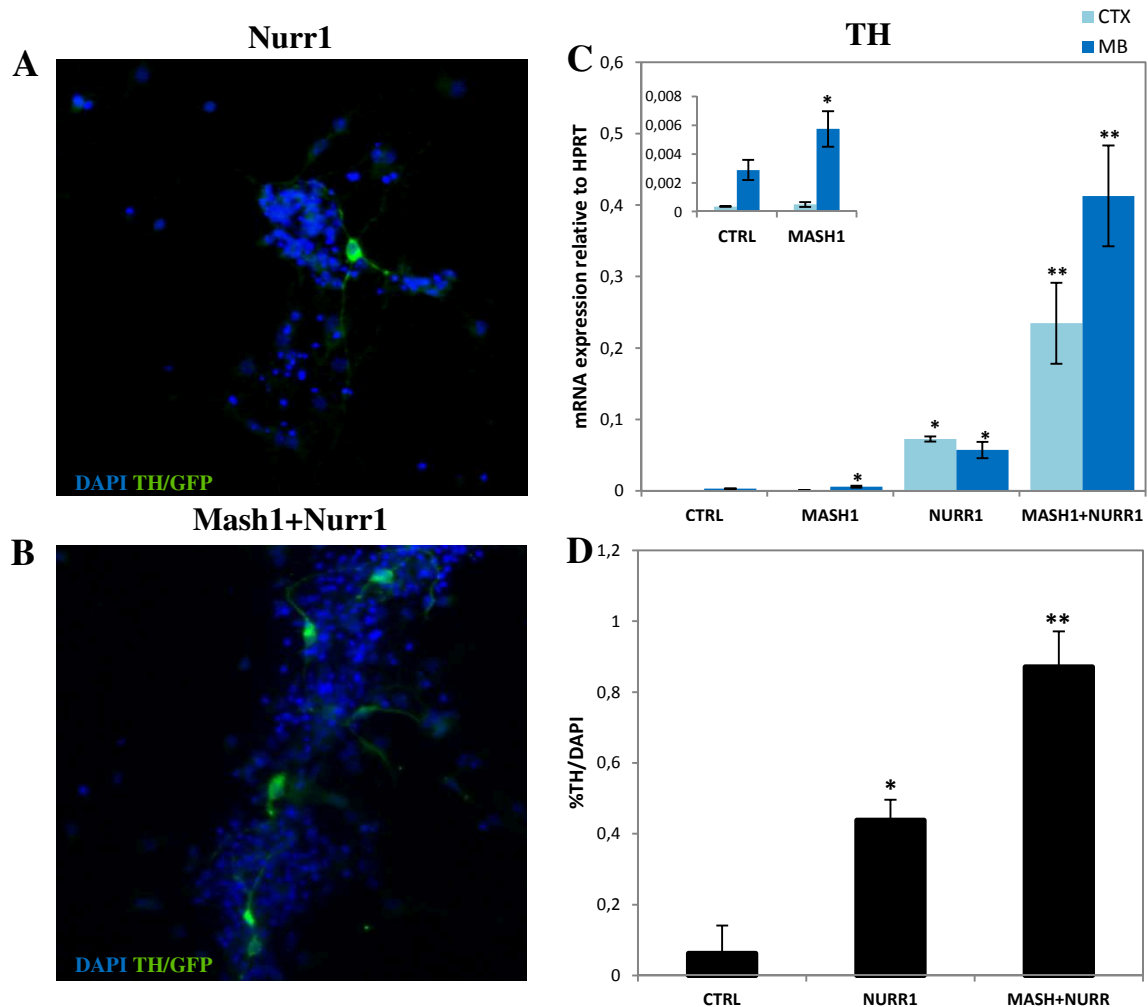


Figure 16. Mash1 and Nurr1 enhance TH expression. (A,B) Immunofluorescence showing TH expression in treated cells, counts are displayed in the graph in (D). (C) Double infection with Mash1 and Nurr1 lentiviruses results in a massive increase in TH mRNA expression both in cortex (CTX) and in midbrain (MB) derived cells.

Remarkably, these results were obtained not only with midbrain-derived cells but also with those derived from the cortex, a brain area that normally does not give rise to DA lineage cells. This data could indicate a specific cooperative effect on TH expression that is independent from regional commitment.

The immunofluorescence for the GFP, expressed under the control of TH promoter confirms the effects of the co-expression of Mash1 and Nurr1 that result in significantly higher TH expression when compared with Nurr1 alone.

In control cultures and Mash1 treated cultures I could not detect expression of TH⁺ cells thus confirming that Mash1 alone is not sufficient to induce DA fate (Fig.16). Furthermore, when I counterstained differentiated cells with an antibody that recognize Tuj1, a marker expressed only in post-mitotic neurons, I could confirm that the majority of TH⁺ cells were post-mitotic neurons overexpressing both Nurr1 and Mash1 (Fig. 17).

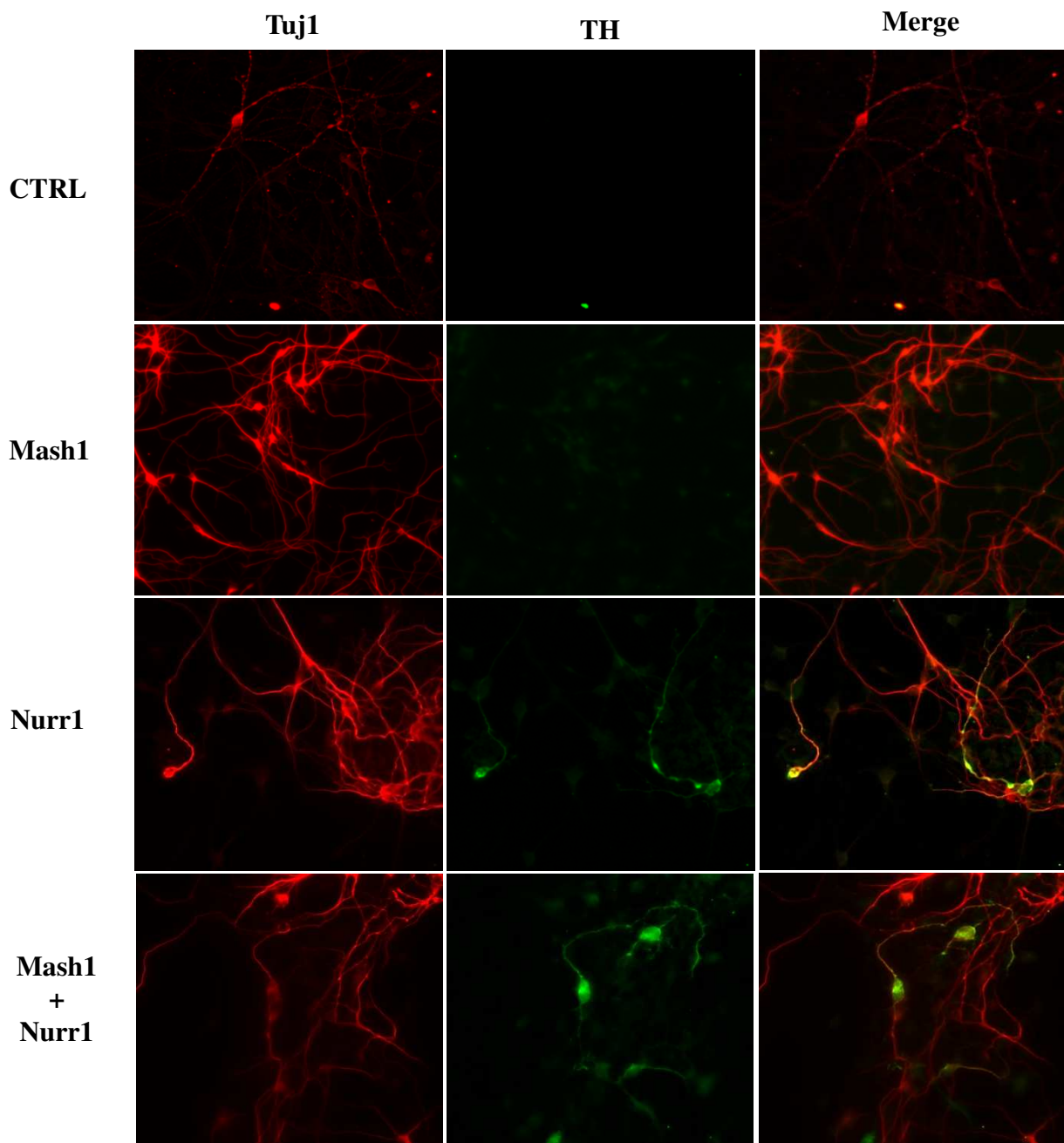


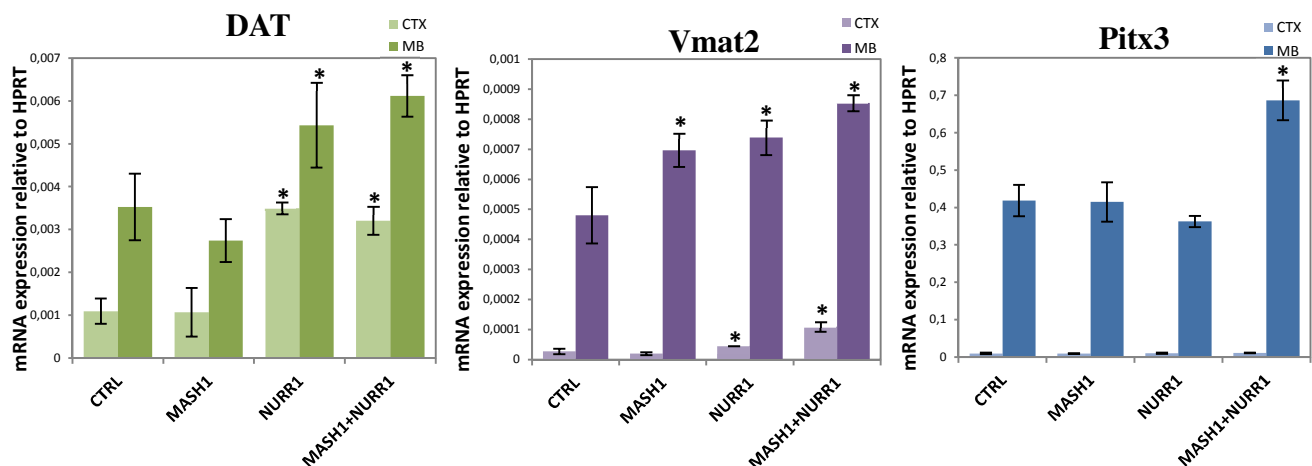
Figure 17. Generated cells are post-mitotic. Immunofluorescence shows that treated cells express the same levels of the post-mitotic neuronal marker Tuj1.

3.2.3 Mash1 and Nurr1 overexpression increases the expression of dopaminergic markers

To assess the identity of the differentiated cells, I also analyzed the expression of genes that are distinctive of the dopaminergic phenotype, such as the dopamine transporter (DAT), the vesicular monoamine transporter Vmat2 and Pitx3, a specific marker of midbrain dopaminergic neurons (Fig.18-A). Interestingly, I observed a marked difference between cortex and midbrain-derived cultures. The latter show higher levels of dopaminergic transcripts than the former condition, suggesting that the appropriate cellular context allows a more extensive functional maturation of DA neurons. It is interesting to note that despite these differences, the overexpression of single transcription factors or the co-overexpression of both TFs have shown a similar trend in both types of cultures. Pitx3 and Vmat2 expression showed a slight increase when cells are co-treated with Nurr1 and Mash1, while a significant increase of DAT levels was observable only with Nurr1 overexpression since co-treatment with Mash1 had not any additive effects. Pitx3 expression, almost absent in cortex-derived cultures, does not seem to undergo significant variations, with the exception of a significant increase in midbrain-derived cultures co-treated with Nurr1 and Mash1. Nevertheless, the relative levels of expression of all observed markers are low. One possible explanation of these phenomena could be that although treated cells are post-mitotic, they are still completing the final phase of maturation.

Moreover, to exclude a differentiation towards other phenotypes, I checked the mRNA expression of non-dopaminergic genes such as *Tryptophan hydroxylase 1* (Tph1) for serotonergic phenotype, the adrenergic marker *Phenylethanolamine N-methyltransferase* (PNMT) and the *Dopamine- β -hydroxylase* (DBH) which is a marker for noradrenergic neurons. As showed in Fig.18-B, the expression of these markers was found significantly low, either in cortex- and midbrain-derived cells, indicating that the related phenotypes were not induced by the treatment (Fig.18-B)

A Dopaminergic genes



B Non-dopaminergic genes

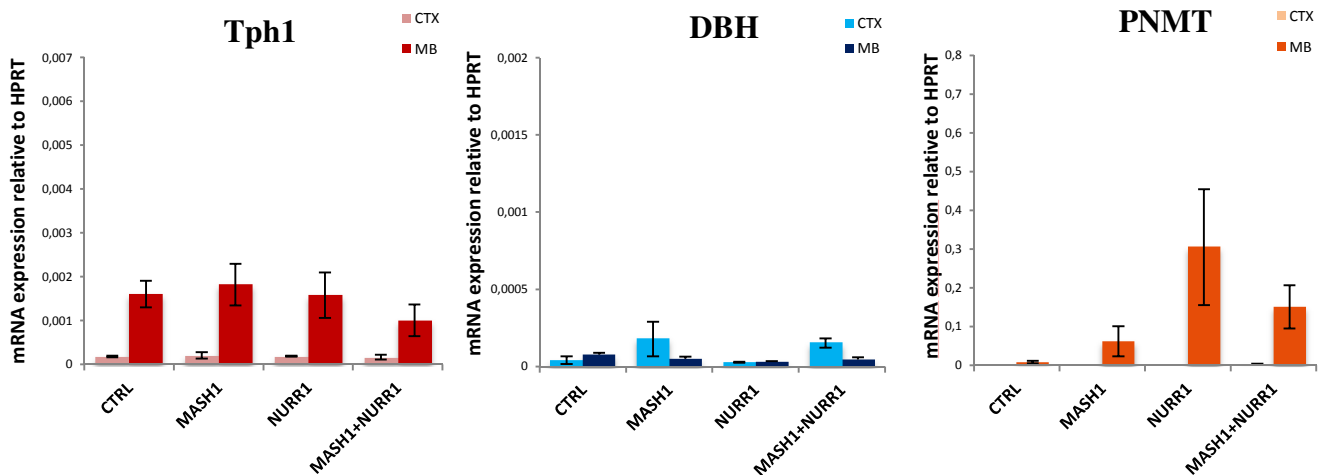


Figure 18. mRNA expression of dopaminergic and non-dopaminergic genes. In the charts are compared the levels of dopaminergic genes in cortex (CTX) and midbrain (MB) derived cultures after treatment with Nurr1 and Mash1. Transcripts for other phenotypes (B), such as serotonergic

3.2.4 Mash1 has a positive effect on Nurr1-induced expression of TH

Altogether the above described data seem to suggest a cooperative action of Mash1 and Nurr1 on the expression of TH. Since Nurr1 is a direct activator of TH transcription, I wanted to assess the effect of exogenous Nurr1 on TH expression in absence or presence of Mash1. For this purpose, I immunolabeled differentiated cells with an antibody directed versus Flag, an epitope that is fused to the N-terminal of the Nurr1 transgene. Thus I have been able to visualize the presence of Nurr1 in the nuclei of TH⁺ cells.

Unexpectedly, although Nurr1 was present in the nucleus of treated cells, not all the Nurr1⁺ cells expressed also TH. Interestingly, when Mash1 was co-expressed with Nurr1, I observed a significantly increase in the number of Nurr1⁺ cells that were also positive for TH. This effect is in favor of my working hypothesis regarding the cooperation between Mash1 and Nurr1, and suggests that Mash1 could modulate the activity of Nurr1 on TH expression.

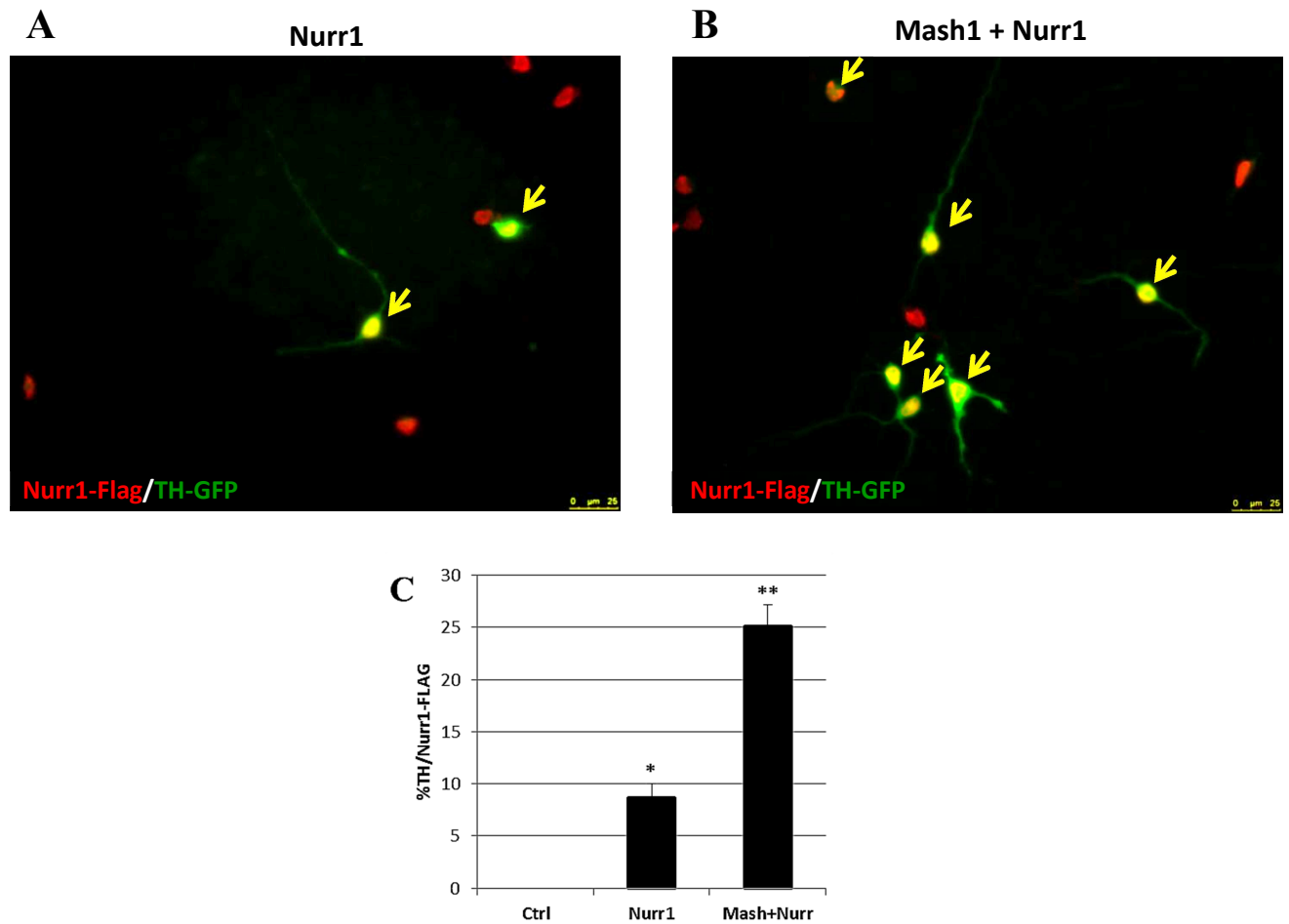


Figure 19. Immunostaining for Nurr1 and TH on treated cultures: Image shows midbrain-derived cultures treated with Nurr1 alone (A) compared with double treated cultures (B). In C are represented cell counts showing the percentage of TH⁺ cells on Nurr1⁺ cells.

3.2.5 The mesencephalic cell line A1 efficiently responds to the overexpression of Mash1 and Nurr1

The heterogeneity and the pre-existence of a regional induction of neurosphere-derived cells can affect the efficiency of the protocol resulting in a low percentage of TH+ neurons as compared on the whole culture. To overcome these difficulties, I repeated the overexpression experiments in the immortalized cell line *mes-c-myc A1* (A1). A1 cells are derived from embryonic midbrain and apparently retain the characteristics of neural progenitors. They are capable to differentiate in neurons and glia (Colucci-D'Amato et al. 1999). The principal advantage of this culture is the homogeneity of the cells and the responsiveness to differentiating conditions with a relative low cell mortality. As shown by qPCR analysis (Fig.20), the co-overexpression of Mash1 and Nurr1 resulted in a major increase in TH expression compared with the expression of the individual transcription factors. This data faithfully replicates those obtained on neurosphere-derived cultures, but in this case the levels of TH are 10-fold higher.

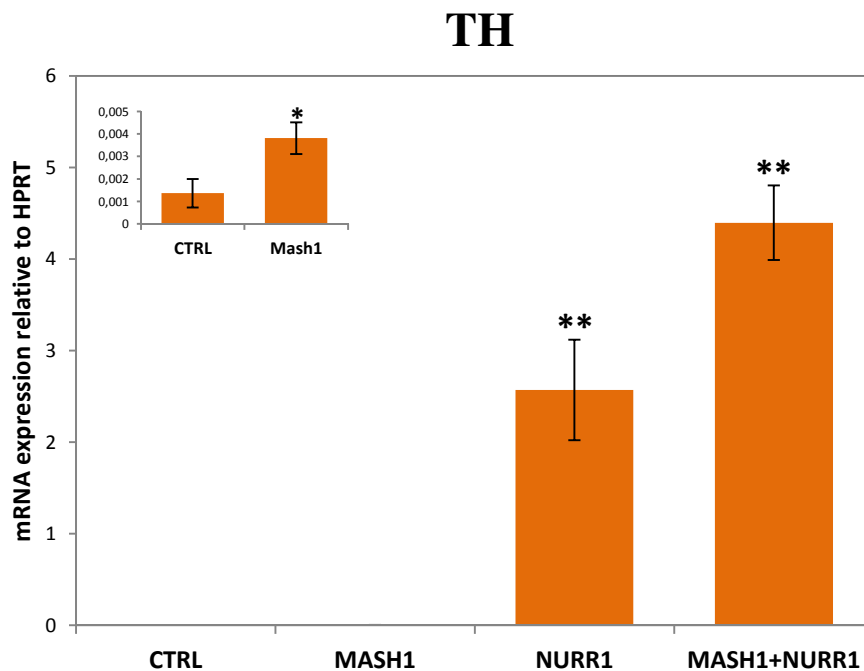


Figure 20. Enhancement of TH expression in A1 cells. Mash1 and Nurr1 co-overexpression enhance TH levels more than Nurr1 alone or Mash1 (small chart on the left) alone.

Also in the A1 cells, the relative expression of dopaminergic genes was enhanced by the double treatment as in the neurosphere-derived cultures (Fig.21). In particular, Vmat2 (Fig.21-A) and DAT (Fig.21-C) followed the same trend observed in neurospheres with the former responding to the action of both transcription factors and the latter responding to the action of Nurr1 alone. Pitx3 mRNA (Fig.21-B) responded to individual overexpression of Mash1 and Nurr1 with a ≈ 3 -fold increase compared to untreated control.

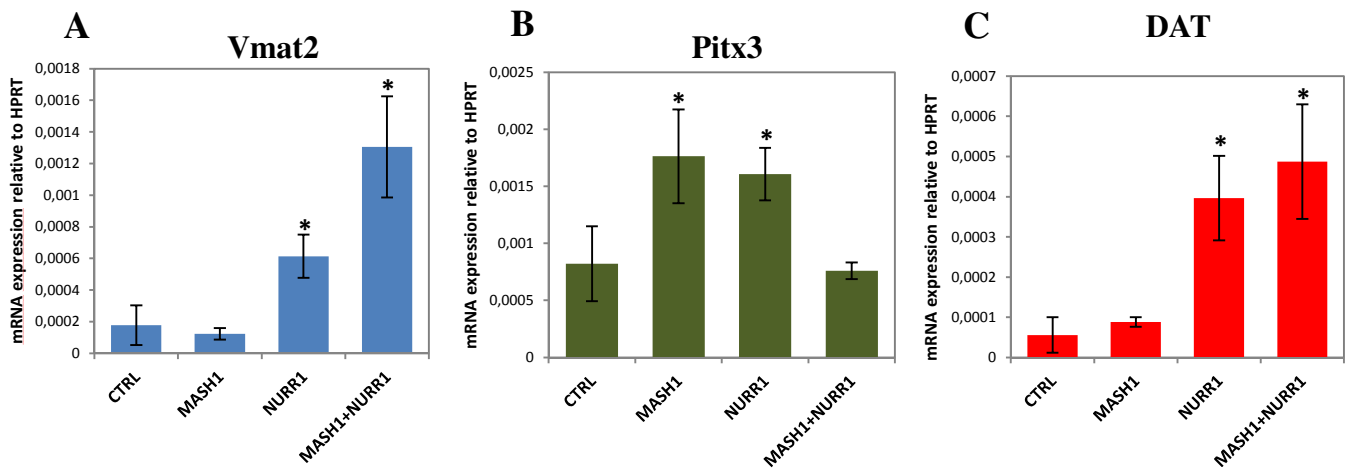


Figure 21. Expression of dopaminergic genes in A1 cells. Co-overexpression of Mash1 and Nurr1 increase the expression of Pitx3(A), VMAT2 (B) and DAT (C)

3.3 Mash1 binds to the TH promoter

Altogether the data showed a new cooperative mechanism of Mash1 and Nurr1 in the differentiation of dopaminergic neurons. Their combined action is most evident on the expression of TH rather than other dopaminergic markers indicating that the two transcription factors could interact to enhance the expression of this gene. Thus, I hypothesized that the two TFs could interact at transcriptional level on the TH promoter. The cooperation can be explained by two models (Fig.22): the first model (Fig.22-A) provide for a cooperation between Mash1 and Nurr1 through a direct interaction in the context of the transcriptional complex present on Nurr1 binding site (NBRE); the second model (Fig.22-B) is based on previous literature data showing the binding of Mash1 to specific *enhancer* sequences (E-box) modulate the activity of its target promoters (Nakada et al. 2004; Kageyama et al. 2005; Poitras et al. 2007). Considering these literature data, the hypothesis showed in Fig.21-B appears to be more suitable.

According with these hypothesis, I proceeded to demonstrate first the presence of putative binding sites for Mash1 by analyzing the sequence of TH promoter, then I verified the binding of Mash1 with these sites or, indirectly, with NBRE, taking advantage of *chromatin immunoprecipitation* (ChIP) experiments.

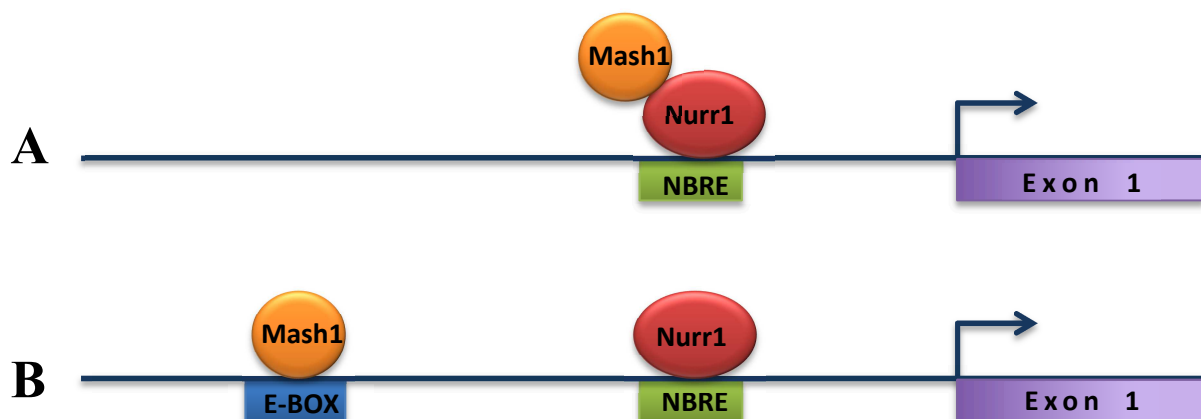


Figure 22. Hypothetical models to explain Mash1 and Nurr1 cooperation. Schematic representation of hypothetical mechanisms of action of Mash1 and Nurr1 for the activation of TH expression: **A)** Mash1 interacts physically with Nurr1 within the transcriptional complex present on NBRE binding site to modulate the activity of the complex; **B)** Mash1 binds an enhancer sequence (E-box) distal from NBRE to modulate Nurr1-dependent transcription of TH *via* an indirect mechanism.

3.3.1 The TH promoter contains three putative E-box for Mash1

As pointed out above, Mash1 is known to bind a conserved motif called E-box (CANNTG) that functions as enhancer in many promoters. To verify their presence on TH promoter, I retrieved a 8101bp sequence on the NCBI database (<http://www.ncbi.nlm.nih.gov/nucleotide/>; ID: JN963248.1) which comprises the 5'upstream sequence of mouse TH gene and part of the first exon. According with Kim *et al.* (Kim et al. 2003d), the transcription start site (TSS) is positioned at 7986bp on the sequence.

I therefore analyzed this sequence availing of the Regulatory Sequence Analysis Tool (RSAT; <http://rsat.ulb.ac.be/rsat/>; Turatsinze et al. 2008), a software able to scan a promoter sequence to find regulatory elements specific for a given TF. The software uses a position weight matrix (PWM) to analyze the statistical significance of retrieved sequences. For the analysis, I retrieved the Ascl2_1 (ID PB0003.1) matrix on Jaspar core database (<http://jaspardev.genereg.net/>; Mathelier et al. 2014; Fig.22). Subsequently I chosen the three sequences with lowest P-value (i.e. the highest significance) out of 501 sequences resulted from the analysis. These sequences, which I called TH1, TH2 and TH3, are located respectively at -2800 bp, -4700 bp and -7700 bp from the TSS (Table 1).

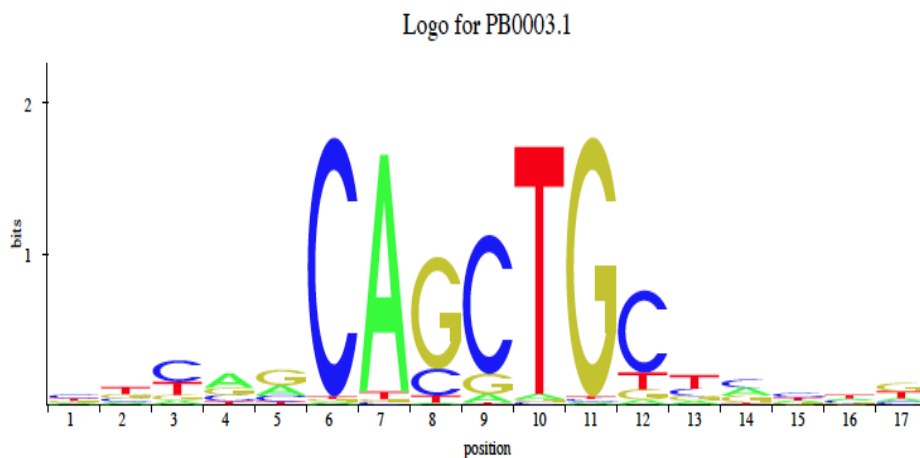


Figure 23. Position weight matrix for Mash1. Graphical representation of the average binding site for Mash1, the height of each base is correlated with the conservation in that determined position.

Table 4. RSAT analysis of TH promoter for Mash1 binding sites.

#seq_id	start	end	sequence	weight	Pval	ln_Pval	sig\
TH	-2923	-2907	TTTAGCAGCTGCAGCTG	11,4	0,00000033	-14,924	6.481\
TH	-4690	-4674	GACCGCAGCTGGTGCTG	8,5	0,000031	-10,382	4.509\
TH	-7830	-7814	AGCAGCAGCTGTCATGA	8,1	0,000047	-9,965	4.328\
TH	-2367	-2351	TAGGCCAGGTGCTACTT	7,2	0,00011	-9,115	3.959\
TH	-7084	-7068	GGCAGCAGGTGTTAGAG	7	0,00014	-8,874	3.854\
TH	-2520	-2504	TCTACCAGCTGTCTCTC	6,8	0,00016	-8,74	3.796\
TH	-4728	-4712	ACTTTCAGCTGCCAAT	6,7	0,00018	-8,623	3.745\
TH	-7833	-7817	CATAGCAGCAGCTGTCA	6,4	0,00023	-8,377	3.638\
TH	-4756	-4740	CTGATCAGCAGCTCTTG	6,3	0,00025	-8,294	3.602\
TH	-4645	-4629	CGAGGCTGCTGCTCCAT	6,3	0,00025	-8,294	3.602\
TH	-3064	-3048	CTTCCCAGCTACTCCTC	6,3	0,00025	-8,294	3.602\
TH	-5696	-5680	CACTCCACCTGTCACAT	6,2	0,00027	-8,217	3.569\
TH	-4753	-4737	ATCAGCAGCTCTTGCCT	6,2	0,00027	-8,217	3.569\
TH	-4687	-4671	CGCAGCTGGTGCTGTCT	6,1	0,00029	-8,146	3.538\
TH	-3388	-3372	GGCAGCAGCTTCCACAC	5,9	0,00034	-7,987	3.469\
TH	-2554	-2538	GCCACAGATGCTTTAG	5,8	0,00037	-7,902	3.432\
TH	-6392	-6376	CTCCACACATGCAGAAG	5,8	0,0004	-7,824	3.398\
TH	-7647	-7631	AGGCCCAGCTGTGAAAT	5,2	0,00059	-7,435	3.229\
TH	-1315	-1299	TCTGGCTCCTGCTAATA	5,2	0,00059	-7,435	3.229\
TH	-3379	-3363	TTCCACACCAGCCGCAC	5,1	0,00063	-7,37	3.201\

3.3.2 Mash1 binds two sites on TH promoter

To verify the binding of Mash1 with the putative sequences, I performed a ChIP assay on whole chromatin isolated from E12.5 mouse midbrains. Indeed at this stage, in which DA progenitors are switching to post-mitotic phase, both Mash1 and Nurr1 are co-expressed in midbrain; nonetheless E12.5 is the stage in which I isolated NSCs for the experiments showed above. The demonstration of the *in vivo* binding of Mash1 with TH promoter would firstly explain the results obtained in neurospheres and finally would provide a strong indication that this mechanism is involved in the switch of proliferating DA progenitors to post-mitotic phase. Thus, I immunoprecipitated (IP) the isolated chromatin using an anti-Mash1 antibody or a nonspecific IgG as mock control and I verified the enrichment of Mash1 by a Western Blot experiment (Fig.23-A) confirming the efficiency of the reaction. I therefore performed a semi-quantitative PCR analysis on isolated DNA to verify the enrichment of the sequences in the IP. Dll1, a known Mash1 target (Castro et al. 2006), was used as positive control.

Noticeably, Mash1 was found to bind TH1 and TH2 sequences with a 9-fold and a 7-fold increase respectively as compared with the mock (Fig.23-B) while no significant enrichment of TH3, the more distal binding site, was found.

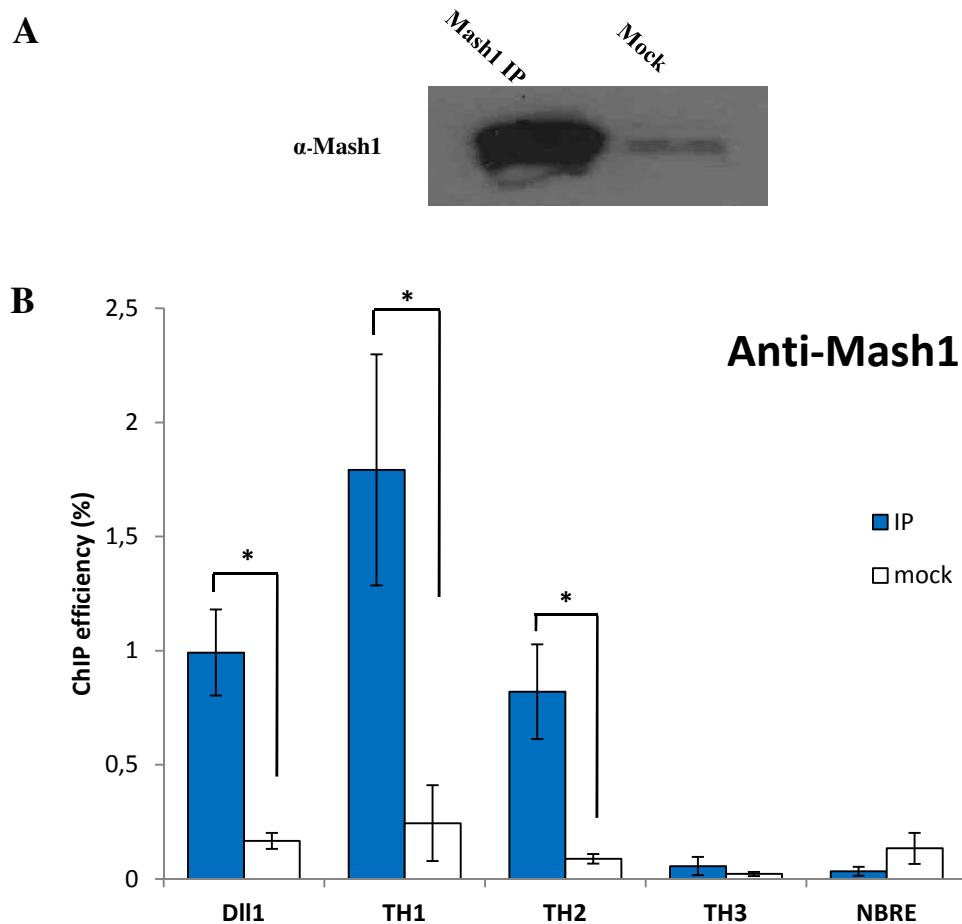


Figure 24. ChIP analysis of the binding of Mash1 to TH enhancers: A) Western Blot comparing Mash1-IP with mock-IP, evidencing the high enrichment of the first. B) Histogram represents enrichment of Mash1 on predicted binding sites of TH promoter. IP efficiency was calculated as the ratio of precipitated sequence over total amount of sequence in the input chromatin.

I also analyzed whether Mash1 was also enriched at the NBRE, the specific binding site for Nurr1 (Fig.23-B; Volpicelli et al. 2012), the IP showed that Mash1 was not enriched. This result indirectly indicates that there is not a physical interaction between Mash1 and Nurr1.

This observation was also supported by data I obtained in the lab using a reporter assay in which the NBRE sequence is cloned upstream of the luciferase gene. Upon co-transfection of the reporter with plasmids encoding for Nurr1 and Mash1 in the

immortalized cell line HeLa and in the more context-specific A1 cell line I could not observe a significant increase of reporter activation (Fig.24).

Altogether these data demonstrate that Mash1 seems to enhance Nurr1 transcriptional activity through the binding to the TH promoter rather than through a direct interaction with Nurr1.

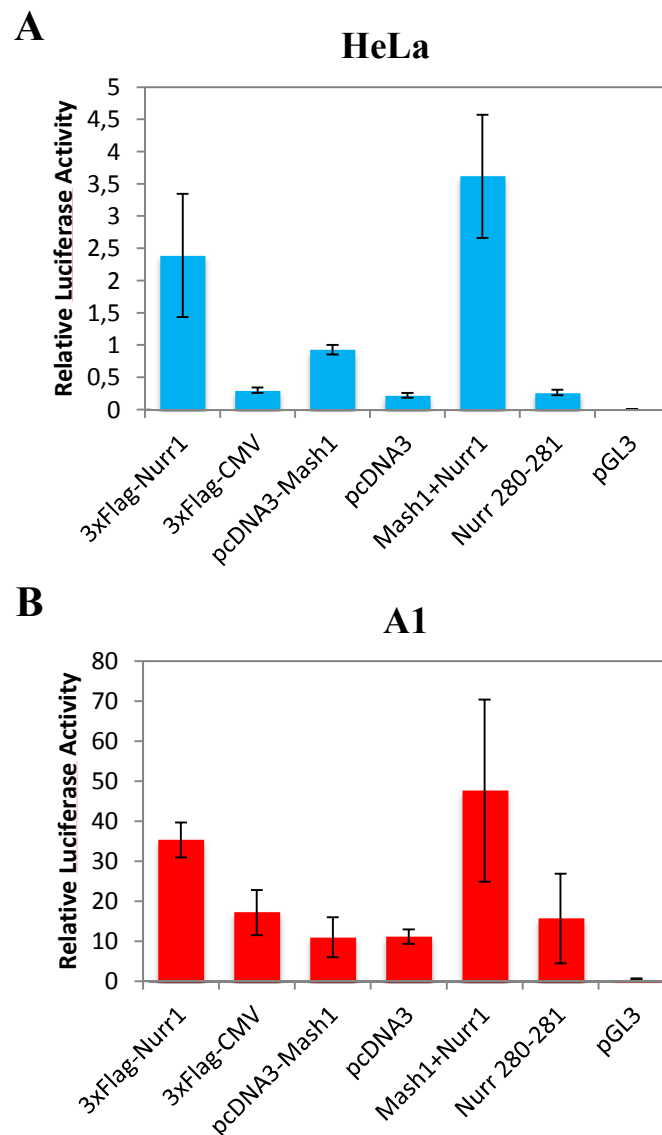


Figure 25. NBRE luciferase assay. Relative reporter activity of NBRE following transfection of Mash1 and Nurr1 in HeLa cells (**A**) and A1 cells (**B**). The binding-defective mutant Nurr280-281 was used as negative control and the activity of each gene was compared to relative empty vectors and to empty reporter vector.

4. DISCUSSION

The brain is a very sophisticated machine whose fine architecture is the result of an orchestrated developmental plan, which is necessary to drive a single totipotent cell to give rise to a functional organism. Since the brain is composed of about 10^{11} neurons with different specific and integrated functions, many efforts have been made to understand how, during development, single classes of neurons acquire their particular phenotype and how their function is then integrated into a complex environment.

During my PhD thesis, my research has been aimed to untangle the molecular events driving the development of the neurons producing the catecholamine dopamine (DA). Dopaminergic neurons are an anatomically and functionally heterogeneous class of cells, with a broad distribution. The most prominent source of DA neurons is the midbrain where they are organized in three principal nuclei. As outlined in the introduction, of particular interest are the neurons residing in the *substantia nigra* and in the *ventral tegmental area* which give rise to the *nigrostriatal pathway* and the *mesocorticolimbic pathway*. Alterations of these circuits are involved in serious neurological and psychiatric diseases.

I have already discussed how the development of midbrain dopaminergic (mDA) neurons is a complex multi-step process in which cell-intrinsic factors as well as environmental cues are integrated in an accurate space-time sequence to define their phenotypic and functional specificity. Many researches in the last decades have been aimed to characterize the molecules involved in this process, leading to the identification of a number of transcription factors (TFs) able to orchestrate induction, specification, differentiation and maturation of mDA neurons. Nevertheless the hierarchy of network interactions underlying their differentiation still needs to be further elucidated.

A literature survey suggested to me to focus on the transcription factors Nurr1 and Mash1, which could be involved in the switch from the mitotic specification stage to the post-mitotic differentiation phase.

Previous works from our laboratory and others have well established that the orphan nuclear receptor Nurr1 is expressed in mDA post-mitotic progenitors starting from E10.5 and its expression persists in the adult brain. Its absence leads to a specific loss of DA neurons (Zetterström et al. 1996; Saucedo-Cardenas et al. 1998). Indeed, Nurr1 is a direct regulator of genes essential for dopaminergic function, such as TH, the dopamine transporter DAT and the vesicular monoamine transporter, Vmat2 (Sacchetti et al. 2001; Kim et al. 2003b; Colebrooke et al. 2006).

The bHLH factor Mash1 (Ascl1), is a well-known proneural factor since it is essential for neuronal specification in early developmental stages in a broad range of brain areas (Kageyama et al. 1997; Bertrand et al. 2002). Its expression is detectable in the midbrain from E9-E10 until the early post-mitotic phase (Vernay et al. 2005). In addition to the generic neurogenic action, several lines of evidence have shown that it is also involved in specifying neuronal subtype identities. Interestingly, Mash1 is co-expressed with Nurr1 in a subset of AHD2⁺ DA progenitor cells in early ventral midbrain (Park et al. 2006a) while Mash1 and Nurr1 have been shown to be important in the reprogramming of somatic cells into dopaminergic neurons (Caiazzo et al. 2011).

Given these observations, the present work was driven by the hypothesis that Mash1 and Nurr1 may cooperate during the dopaminergic lineage commitment, in the switch from the specification phase to the post-mitotic differentiation phase, triggering the molecular events leading to the correct development of fully differentiated midbrain DA neurons.

Following the isolation of neural stem/progenitor cells (NSCs) from E12.5 mouse midbrain and cortex using the neurosphere assay, under the effect of the mitogens bFGF and EGF, I was able to select only proliferative multipotent neural progenitors, according to results from other laboratories (Reynolds and Weiss 1992; Rietze and

Reynolds 2006). Hence, using functional assays I have assessed the identity of these cells.

In first instance, my results demonstrate the persistent ability of the cells to self-renew through serial passages, and gave rise to secondary spheres. The higher proliferation rate that I observed in cortex-derived cells, a seven-fold increase, when compared to midbrain-derived cells, which showed a three-fold increase, suggested a minor proliferative capacity of the latter. Nevertheless this proliferative trend was stable during serial passages, a feature that demonstrates the self-renewal of the cells. That cortical precursors show a higher proliferation rate in my neurosphere-derived cell cultures is not surprising, since also *in vivo* the formation of the cortex, in mice and even more in humans, is consequent to the expansion of a precursor population (Chenn and Walsh 2002). In parallel experiments I have demonstrated that both cortex- and midbrain-derived cells stably express neuronal progenitor markers, such as Nestin and Sox2, during passages while the post-mitotic neuronal marker β III-tubulin (Tuj1) shows a dramatic decrease after the first passage and remains stably low-expressed. These results indicate that both precursor populations have immature properties. I also demonstrated the multipotency of neurosphere-derived cells, upon mitogens withdrawal and serum addition, by showing that they are able to give rise to the three types of neural lineages, that are neurons, which are positive for Tuj1, GFAP-positive astrocytes and oligodendrocytes expressing Olig2. In my experimental conditions also the relative representation of neurons (3%), astrocytes (62%) and oligodendrocytes (1,3%), either in cortex- and midbrain-derived cells, is consistent with literature data (Reynolds and Weiss 1992; Rietze and Reynolds 2006).

Once I have ascertained the multipotent identity of the cells, my aim has been directed to push neurosphere-derived cells towards DA fate establishing a differentiation paradigm. Therefore I have derived neurospheres from TH-GFP mice, which have the great advantage of enabling the identification, by immunofluorescence, of cells that differentiate into DA neurons, since expression of the fluorescent reporter is driven by the TH promoter.

As shown in the result section, cells exposure to the action bFGF and Sonic Hedgehog (Volpicelli et al. 2004b) followed by the differentiation phase. Overexpressing, in single or double-combination, Mash1 and Nurr1 availing of inducible lentiviral vectors appears a reliable system to evaluate the effect of these genes on the expression of known dopaminergic markers and their hierarchical position in the differentiation network.

That Nurr1 overexpression leads to an increase in TH expression was expected, from previous data in the laboratory and other published work while a major increase of TH transcripts led by co-overexpression of Mash1 was a new interesting result. Notably, this effect observable in both midbrain-derived as well as in cortex-derived cells, strongly indicate that there in these culture there is a relative loss of the regionalization specificity or that the effect of both factors on TH expression is regionalization-independent and does not require a specific dopamine-precursor cellular context.

On the contrary the finding that other markers, normally expressed only in mature DA neurons, such as DAT, Vmat2 and the midbrain-specific Pitx3, is modified by Mash1 and Nurr1 overexpression, albeit at low levels, only in midbrain-derived cells, is rather in favor of the hypothesis that only a proper cellular context allows these two transcription factors to sustain maturation of DA neurons. However, the low expression level of these markers shows that the cells are immature or still completing their differentiation at the stages analyzed.

These observations prompted me to inquire how Mash1 and Nurr1 could cooperate in defining the DA phenotype, through the specific activation of TH expression. My hypothesis is strengthened by the observation that the overexpression of Mash1 alone led to a two-fold increase in TH expression only in midbrain-derived cells, where endogenous Nurr1 is present. Consistent with these observations, overexpression of the two TFs in the more stable mesencephalic cell line A1 led to comparable effects both on TH expression and on DA markers.

The immunochemistry experiments confirmed that the effect of Mash1 and Nurr1 co-expression regulates the proper DA phenotype since TH-GFP⁺ cells are significantly increased in double-treated cultures compared with Nurr1-transduced cells. The Nurr1- and double-treated cells are undoubtedly post-mitotic since by Tuj1 immunostaining they have been found positive for the β -III Tubulin, a characteristic marker of post-mitotic neurons. Intriguingly, the number of Tuj1⁺ cells is comparable between the single and double-treated cells, suggesting that the double infection did not further affect the number of neurons. Surprisingly not all the cells expressing the exogenous Nurr1 have been found positive by TH immunostaining; however, I show that the number of Nurr1⁺/TH⁺ cells is increased by the co-expression of Mash1. This observation is consistent with a cooperative mechanism between the two TFs and raises the hypothesis that Mash1 could enhance the Nurr1-mediated transcription of TH, while it doesn't have *per se* a direct transcriptional effect on the latter.

This hypothesis can underlie two alternative models:

1. Mash1 binds directly the transcriptional complex formed by Nurr1 on the NBRE sequence and modulates its activity in a direct manner.
2. Mash1 binds to probably distal enhancer sequences modulating Nurr1 activity in an indirect way, i.e. by recruiting chromatin-remodeling factors.

Mash1 is indeed known to bind specific sequences (CANNTG) called E-box to modulate the transcriptional activity of a number of genes (Castro et al. 2006). Thus, I screened the TH promoter for the presence of putative binding sites by using a bioinformatics model which avails of position weight matrices (PWM) to find probable matches on a given input sequence. This analysis confirmed the presence of three significant binding sequences, TH1, TH2 and TH3 positioned respectively at -2800 bp, -4700 bp and -7700 bp from the transcription start site.

Their presence on the normal TH promoter *in vivo* was confirmed by chromatin immunoprecipitation (ChIP) experiment on E12.5 midbrain-derived chromatin. There is a modest enrichment of Mash1 on TH1 and TH2 binding sites but not in the more distal TH3 site.

This experiment supported the hypothesis of an indirect effect of Mash1 on Nurr1-induced activation of TH transcription but did not exclude a direct cooperation between the two TFs. I examined this aspect evaluating the enrichment of Mash1 on the specific Nurr1 binding site (NBRE), by a luciferase assay. The results are in contrast to a direct cooperation model, since Mash1 does not show a significant enrichment on NBRE *in vivo* and the co-expression with Nurr1 does not have any additive effect on the expression of luciferase as compared with Nurr1 alone. I could thus conclude against the possible direct cooperation of Mash1 and Nurr1 on the NBRE site.

In summary, my results show the existence of a cooperative effect of Mash1 and Nurr1 on the differentiation of NSCs towards dopaminergic phenotype. The combined action of the two TFs, drive the cells in the post-mitotic phase and leads to an increase in the expression of the rate-limiting enzyme TH in cortex- and midbrain-derived NSCs. The effect is observable, at low levels, also on the expression of more mature DA markers as DAT, Vmat2 and Pitx3. In particular Mash1 enhances Nurr1-induced activation of TH transcription by binding two distal enhancers on the TH promoter rather than by a physical interaction with Nurr1. This is consistent with a permissive rather than instructive role of Mash1 in the development of DA neurons.

A possible hypothesis to address the nature of this cooperative mechanism is suggested by a recent publication which shows that Mash1 is able to act as a “pioneer factor” by recognizing a repressed state of the chromatin of a number of promoters and allowing the access of specific TFs to activate the transcription (Wapinski et al. 2013). Further experiments will be needed to assess a presumed epigenetic role of Mash1 in TH transcription.

Altogether, these data shed further light on the molecular mechanisms underlying the differentiation of dopaminergic neurons pointing out the vision of a complex hierarchic mechanism which is strictly controlled, at given time points and at different levels, by the interplay of different factors rather than just a sequential transcriptional activation.

A key challenge in stem cell biology is indeed to understand how the different cell-fate options are selected in a heterogeneous population and how the adoption of a given lineage-decision is coupled to appropriate regulation of the alternative genetic pathways. Clearly, while we assume that a gene can only have an “on” and an “off” state, in reality gene expression can be graded giving rise to a large number of combinations. A new vision, highlighted by recent studies, considers the genetic networks associated with key properties, such as self-renewal or differentiation, as a landscape, that can exist in a limited number of solutions or “states”. In this way the nature of stem cells relationship to commitment, differentiation and lineage selection can be described as a transition through stable states, mathematically defined as *attractors* (Enver et al. 2009). From this point of view the differentiation towards a defined lineage can be interpreted as a transition from a stochastic phase to a deterministic phase. In other words, the casual transition to a stable genetic state may activate a hierarchic cascade of molecular events leading to a fully differentiated cell, somehow finding order in chaos or, in the words of Waddington, a *canalization of development* (Waddington 1957).

The new information generated during my thesis will be useful not only for our understanding of transcriptional regulatory cascade underlying the midbrain DA neuron development, but also for future *in vitro* generation of functional DA neurons from stem and/or progenitor cells for cell therapy of Parkinson’s disease, which is caused by the loss of midbrain DA neurons.

5. REFERENCES

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6. LIST OF ABBREVIATIONS

A

AADC	Aromatic L-amino acid decarboxylase
ADHD	Attention deficit hyperactivity disorder
Ahd2	Aldehyde dehydrogenase 2
AK	Aphakia
ALS	Amyotrophic lateral sclerosis

B

Bcl-XL	B-cell lymphoma-extra large
BDNF	Brain-derived neurotrophic factor
bHLH	basic Helix-Loop-Helix
BMP	Bone morphogenic protein

C

cAMP	cyclic Adenosine monophosphate
CDNF	Conserved dopamine neurotrophic factor
ChIP	Chromatin immunoprecipitation
CNS	Central Nervous System
COMT	Catechol-O-methyl transferase

D

DA	Dopamine
DAR	Dopamine receptor
DAT	Dopamine active transporter
DCX	Doublecortin
DIV	Days in vitro
Dmrt5	Doublesex and mab-3-related transcription factor 5
DOPAC	3,4-dihydroxyphenylacetic acid

E

EAE	Experimental Autoimmune Encephalomyelitis
En	Engrailed
ESC	Embryonic Stem Cells

F

FGF8	Fibroblast Growth Factor 8
FP	Floor plate

G	
GDNF	Glial cell line-derived neurotrophic factor
GFR α	GDNF family receptor alpha
GPe	external Globus Pallidus
GPi	internal Globus Pallidus
H	
HVA	Homovanillic acid
I	
iDA	induced Dopaminergic Cells
iN	induced Neuronal cells
IP	immunoprecipitation
iPSC	induced Pluripotent Stem Cells
L	
L-DOPA	Levodopa
LRRK2	Leucine-rich repeat kinase 2
M	
MAO	Monoamine oxidase
Mb	Midbrain
MCLp	Mesocorticolimbic pathway
MHB	Midbrain-Hindbrain boundary
N	
Ngn1-2	Neurogenin 1-2
NGS	Normal goat serum
NSA	Neurosphere assay
NSC	Neural Stem Cells
NSp	Nigrostriatal pathway
Nurr1	Nuclear Receptor Related 1
O	
OB	Olfactory bulb
P	
PD	Parkinson's disease
Pitx3	Pituitary homeobox 3
PKA	Protein kinase cAMP-dependent
PNMT	Phenylethanolamine N-methyltransferase
PPN	Pedunclopontine Nucleus
Ptc1	Patched1
PWM	Position Weight Matrix

R

RA	Retinoic Acid
RRF	Retrorubral field
RXR	Retinoid X receptor

S

SGZ	Subgranular zone
SHH	Sonic hedgehog
Smo	Smoothed
SN	Substantia Nigra
SNC	Substantia Nigra <i>pars compacta</i>
SNr	Substantia Nigra <i>pars reticulata</i>
STN	Subthalamic Nucleus
SVZ	Subventricular zone

T

TF	Transcription Factor
TH	Tyrosine Hydroxylase
TLR	Toll-like receptor
TSS	Transcription Start Site

V

VM	Ventral Midbrain
Vmat2	Vesicular monoamine transporter
VTA	Ventral Tegmental Area
VZ	Ventricular Zone

W

WT	Wild Type
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